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Owner, by virtue of an invention the title of which is

(בעברית)
(Hebrew)
שימוש בפפטיד ACHE ספציפי כחומר גדילה

(באנגלית)
(English)
USE OF A SPECIFIC ACHE PEPTIDE AS A GROWTH FACTOR

מבקש בזאת כי ינתן לי עליה פטנט. hereby apply for a patent to be granted to me in respect thereof.

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שימוש בפפטיד ACHE ספציפי כחומר גדילה

USE OF A SPECIFIC ACHE PEPTIDE AS A GROWTH FACTOR

Acetylcholinesterase-derived peptides and uses thereof

Field of the invention

The invention is directed to the field of stem cell survival and expansion. Specifically, the invention is directed at the stem cell survival and expansion effects of peptides derived from acetylcholinesterase.

Introduction

Stress insults are associated with rapid and significant changes in blood cell composition. For example, following massive blood loss, or after surgery, the hematopoietic system responds within hours, by an elevation of the white blood cell and platelet counts. However, the mechanisms responsible for initiating this adjustment are not fully understood. Glucocorticoid hormones, known to be elevated under stress, play a leading role in the adaptive reaction of the bone marrow in response to stress. Glucocorticoid hormones induce absolute increases in all hematopoietic lineages, especially myeloid cells. This involves a cascade of events culminating in changes in the proliferation, differentiation and apoptotic events characteristic of each of the hematopoietic cell lineages (for review see Lansdorp, Exp. Hematol. 23, 187-91, 1995). Also, significant changes occur under glucocorticoid hormones in the levels of hematopoietic growth factors controlling the proliferation of stem cells from which blood cells develop.

Hematopoietic stem cells (HSCs) are pluripotent, in that they give rise to all blood cell lineages. These cells migrate during ontogeny to settle

in the bone marrow as a permanent self-renewing source of blood cells. Under normal conditions the vast majority of HSCs are nondividing, but under conditions of development or stress they can undergo clonal expansion and self-renewal (Keller and Snodgrass, J. Exp. Med. 171, 1407-18, 1990). A large number of cytokines and growth factors, such as stem cell factor (SCF), thrombopoietin (TPO), and FLT-3 ligand, are thought to mediate the proliferative capacity of HSCs, through specific receptors -- c-kit, c-mpl and flt3/flk-2, respectively. Alone, their capacity to stimulate proliferation is limited. For example, SCF can maintain survival for a few days *in vitro*, but not the self-renewal of HSCs (Li and Johnson, Blood 84, 408-14, 1994). However, when used in combination, these growth factors acquire a potent co-stimulatory effect. The early phase of adaptation of the hematopoietic system to stress (first 24 hr), requires coordinator(s), such as leu-enkephalin, which modulate the effects of growth factors on stem cells. However, leu-enkephalin is present in the circulation only immediately following the stress insult, whereas the modulation of hematopoiesis continues long after that phase. Therefore, additional long-acting modulators remain to be identified.

The enzyme Acetylcholinesterase (AChE) is expressed in brain tissue, but also in most, if not all, of the mammalian hematopoietic cell lineages. AChE is expressed in many parts of the vertebrate embryo, with a developmentally regulated pattern in specific cell types and tissues during the embryonic and adult stages. AChE diversity is noted in several pathological states, such as Alzheimer's disease, where AChE activity was

shown to decrease, not only in the primary site of the disease, the brain, but also in the hematopoietic system.

It has now surprisingly been found that the C-terminal peptides of AChE-S and AChE-R have independent biological activities. Specifically, it has been found that these peptides promote stem cell survival. It has also been found that these peptides promote stem cell expansion, when used in combination with growth factors. Further, it has been found that such peptides are capable of augmenting hematopoiesis *in vivo*.

Summary of the invention

This invention is directed at a cell growth and/or differentiation regulatory peptide comprising a sequence of about 9 to about 150 amino acids derived from Acetylcholinesterase amino acid sequence. The said sequence preferably contains a region predicted to be rich in beta-pleated sheet structure and turns. Also preferably, the said sequence contains a predicted amphipathic helix structure. In another embodiment of the peptide of the invention, the said sequence is derived from the C-terminal region of acetylcholinesterase.

The said sequence is preferably derived from the readthrough or synaptic variant of acetylcholinesterase, preferably from the mature form thereof. The said sequence is preferably about 20 to about 50 amino acids in length. More preferably, the said sequence is SEQ ID No. 1, SEQ ID No. 2, or SEQ ID No. 3. Still more preferably, the said peptide is SEQ. ID No. 1, SEQ. ID. No. 2, or SEQ ID No. 3.

A peptide of the invention which is a cyclic peptide is also considered to be within the scope of the invention.

The peptide of the invention is preferably synthetic and preferably comprises the amino acid sequence of SEQ ID No. 1, SEQ ID No. 2, or SEQ ID No. 3. More preferably, the peptide has the amino acid sequence denoted by SEQ ID No. 1, 2 or 3. The peptide is preferably linear and synthetic. In another embodiment of the invention, the peptide is cyclic and synthetic.

In one embodiment, the invention provides a peptide capable of promoting cell survival and/or differentiation and comprising the amino acid sequence denoted by SEQ ID No. 1, SEQ. ID. NO. 2 or SEQ ID No. 3, and functional analogues and derivatives thereof.

In another embodiment, the invention provides a peptide of the invention which is a hematopoietic stem cell growth and/or differentiation regulatory peptide. Preferably, said peptide is capable of promoting stem cell survival and/or myeloid and megakaryocytic differentiation and comprises the amino acid sequence denoted by SEQ ID No. 1, SEQ. ID. NO. 2 or SEQ ID No. 3, or functional analogues and derivatives thereof. Said peptide may be either linear or cyclic, and is preferably synthetic.

The invention also relates to a pharmaceutical composition comprising a synthetic peptide of any one of the preceding embodiments of the invention.

The pharmaceutical composition preferably comprises a synthetic peptide comprising the amino acid sequence of SEQ ID No. 1, SEQ. ID. NO. 2, or

SEQ ID No. 3. More preferably, the pharmaceutical composition comprises a synthetic peptide having the amino acid sequence denoted by SEQ ID No. 1, SEQ. ID. NO. 2, or SEQ ID No. 3. The peptide contained within the pharmaceutical composition may be either linear or cyclic, and is preferably synthetic.

The invention provides a pharmaceutical composition according to the invention, for use in regulating hematopoietic stem cell growth, promoting survival of stem cells, differentiation of stem cells, promoting growth of stem cells, and/or promoting the growth-enhancing effect a growth factor on stem cells. The growth factor is preferably GM-CSF, SCF, or TPO.

In a preferred embodiment of the invention, the stem cells are embryonic stem cells, nerve stem cells, epithelial stem cells, mesenchymal stem cells, or hematopoietic stem cells.

The invention provides a pharmaceutical composition comprising a peptide according to the invention use in the treatment of thrombocytopenia, post-irradiation condition, post-chemotherapy condition, or condition following massive blood loss. The invention also provides said pharmaceutical composition for use in inducing synthesis of acetylcholinesterase mRNA and/or promoting the formation of hematon bodies.

In a further embodiment, the invention provides an antibody directed against a peptide of the invention. The antibody is preferably provided for use in diagnosing elevated glucocorticoid level; bone marrow stress, abnormality, dysfunction, or stressed condition, or of increased

platelet count or of brain infarct risk in a mammal. The antibody is preferably directed at a peptide comprising SEQ ID No. 1, SQ ID No. 2, or SEQ ID No. 3. More preferably, the antibody is directed at a peptide which is selected from SEQ ID No. 1, SQ ID No. 2, or SEQ ID No. 3.

In yet another embodiment, the invention provides a method for the diagnosis of elevated glucocorticoid level; bone marrow stress, abnormality, dysfunction or stressed condition, or of increased platelet count or of brain infarct risk in a mammal, comprising obtaining a sample from said mammal, contacting said sample with an antibody of the invention, removing unbound antibody, and detecting the extent of reaction between said antibody and acetylcholinesterase or a fragment thereof present in said sample. The said sample is preferably a serum or bone marrow sample.

In a still further embodiment, the invention provides a peptide thereof for use in *ex vivo* or *in vivo* expansion of hematopoietic stem cells.

The invention also provides a peptide thereof for use in *ex vivo* or *in vivo* promotion of megakaryocytic differentiation of hematopoietic stem cells.

Brief descriptions of the Figures

Fig. 1 A shows a scheme of the *ACHE* upstream gene sequence. Arrow, transcription start site, triangles, conserved transcription factor binding motifs, black (or green, red, or blue) boxes, exons 1, 5, 6 and intron 4' as indicated, white (or pink) boxes, exons 2-4 and introns 2'-3- as

indicated; **Fig. 1 B**, Enrichment of UCB $CD34^+$ cells, **Fig. 1 C**, cytochemical staining of enriched $CD34^+$ cells for AChE catalytic activity in the presence of inhibitors for BuChE and AChE, **Fig. 1 D**, effect of hydrocortisone on the expression of AChEmRNA splicing variants (R, H and S) in UCB $CD34^+$ cells;

Fig. 2 shows the spatiotemporal shifts in the intensity of embryonic AChEmRNA transcripts through blood cell forming tissues, **A**, blood cell-forming organs analyzed. Top left: A sagittal section of a human embryo showing the hematopoietic organs – AGM (aorta-gonad-mesonephros), LIV (liver), SPL (spleen), and BM (bone marrow). Top right: Scheme of gestational shifts in hematopoietic processes; **Fig. 2 B**, A*CHE* gene expression in embryonic tissues;

Fig. 3 A, shows the amino acid sequence and predicted secondary structure of the ARP and ASP peptides, **Fig. 3 B**, effect of ARP (black bars) or ASP (gray bars) peptide, compared to controls (white bars) on survival of $CD34^+$ cells, in combination with the indicated growth factors;

Fig. 4 shows that ARP operates as an autologous inducer of *ACHE* gene expression. Left, in-situ hybridization of representative $CD34^+$ cells treated with ARP, Right, Average labeling densities of AChE mRNA splice variants (S, E, R) versus ARP concentration (top);

Fig. 5 shows that ARP induces stem cell proliferation as measured by BrdU incorporation. **A**, scheme of AChE and BuChE (BChE) genes and AChE splice variants, **B**, Selective susceptibility of AChE-R mRNA in $CD34^+$ stem cells to AS-ODN destruction. **C**, stem cell proliferation in the

presence of the indicated antisense ODNs with GM-CSF and ARP added as indicated;

Fig. 6 shows redundant properties of ARP and SCF;

Fig. 6 A shows cell counts from long-term $CD34^+$ liquid cultures grown in the absence of growth factors (diamonds), in the presence of early-acting cytokines (EAC: IL3, IL6, TPO and FLT3) and SCF (squares), in the presence of EAC + ARP (triangles) or in the presence of EAC + ARP + SCF (circles). Upper left, viable cell count, Upper right, CD34+ cell count, Lower left, colony forming unit count for GM progenitors, Lower right, colony forming unit count for MK progenitors;

Fig. 6 B shows representative photographs of the 28-day liquid cultures detailed in Figure 6A, Upper left, control, Upper right, cultures treated with EAC and SCF, Lower left, cultures treated with EAC+SCF+ARP, Lower right, cultures treated with EAC+ARP;

Fig. 7 shows that ARP has short- and long-term hematological effects *in vivo*. **Fig. 7A** shows that ARP accumulates in the serum under stress. Top: Poinceau-stained polyacrylamide gels, Bottom: detection of ARP and AChE (arrows) in the immunoblot by anti-ARP antibodies. **Fig. 7B** shows that ARP facilitates the stress-induced hematopoietic responses *in vivo*. **Fig. 7C** shows that persistent AChE-R overproduction increases platelet and WBC counts in a dose-dependent manner. Asterisks in Figs 7A and B denote statistical significance ($p \leq 0.05$, ANOVA).

Detailed description of the invention

A number of terms as used herein is defined hereinbelow

-AChE, Acetylcholinesterase;

-AS, antisense;

-AS-ODN, antisense oligodeoxynucleotide;

-ARP, acetylcholinesterase "readthrough" peptide;

-ASP, acetylcholinesterase "synaptic" peptide;

-BFU, burst-forming units;

-B, erythroid bursts;

-Blast or bl, blast cell (colonies);

-BuChE, Butyrylcholinesterase;

-CFU, colony-forming units;

-CSF, colony-stimulating factor;

-"derived from" acetylcholinesterase, the term "derived from", when referring to acetylcholinesterase or to equivalent terms, in the context of this application is intended to mean amino acid sequence corresponding to amino acid sequence of acetylcholinesterase protein, or to predicted amino acid sequence of the open reading frame of an acetylcholinesterase splice variant mRNA. This includes sequences identical to acetylcholinesterase sequence, and sequences having one or more deletions, additions, and/or substitutions, preferably of conservative nature, i.e., changes that are not expected to change the overall structure of the peptide. However, any change that does not affect the function or activity of the peptide or that increases same is contemplated to be within the scope of the invention;

-EAC, early-acting cytokines, these are preferably IL-3, IL-6, Flt3, thrombopoietin, but may also comprise others such as soluble IL-6 receptor;

-GEMM, granulocyte –erythrocyte –macrophage -megakaryocyte (colonies);

-GM, granulocyte-macrophage (colonies);

-GST, glutathione-S-transferase;

-HSC, hematopoietic stem cells;

-Mix, mixed hematopoietic colonies;

-MK, Megakaryocyte (colonies);

-ODN, oligodeoxynucleotide;

-ORF, open reading frame;

-RT, room temperature;

-UCB, umbilical chord blood;

-UTR, untranslated terminal region;

-WBC, white blood cells.

This invention relates to peptides derived from the open reading frame of the acetylcholinesterase mRNAs. The peptides of the invention are useful, *inter alia*, in upregulating AChE mRNA, enhancing growth and/or differentiation of stem cells.

A number of methods of the art of molecular biology are not detailed herein, as they are well known to the person of skill in the art. Such methods include site-directed mutagenesis, PCR cloning, expression of

cDNAs, analysis of recombinant proteins or peptides, transformation of bacterial and yeast cells, transfection of mammalian cells, and the like. Textbooks describing such methods are e.g., Sambrook *et al.*, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory; ISBN: 0879693096, 1989, Current Protocols in Molecular Biology ,by F. M. Ausubel, ISBN: 047150338X, John Wiley & Sons, Inc. 1988, and Short Protocols in Molecular Biology, by F. M. Ausubel *et al.* (eds.) 3rd ed. John Wiley & Sons; ISBN: 0471137812, 1995. These publications are incorporated herein in their entirety by reference. Furthermore, a number of immunological techniques are not in each instance described herein in detail, as they are well known to the person of skill in the art. See e.g., Current Protocols in Immunology, Coligan *et al.* (eds), John Wiley & Sons. Inc., New York, NY.

AChE pre-mRNA undergoes alternative splicing, which generates, at the translational level, three variants of the AChE protein (Ben Aziz Aloya *et al*, Proc. Natl. Acad. Sci. USA 90, 2471-5, 1993). The best known transcript is AChE-S mRNA, formed by splicing of exon 4 to exon 6, which yields the principal "synaptic" isoform, found in brain and muscle (Soreq *et al.*, Proc. Natl. Acad. Sci. USA 87, 9688-92, 1990). Translation of this mRNA, results in a C-terminal extension of the common core domain of 534 amino acid residues by a 40 residue peptide, containing the cysteine that is involved in dimerization. The second splicing option, which generates the erythrocytic transcript (AChE-E), is based on splicing of exon 4 to exon 5, which encodes a different 40 residue peptide at the

C-terminus. This latter peptide is subsequently cleaved at residue 14 (number 557 from the N-terminus) and forms a glycophospholipid linkage that may be integrated into erythrocyte membranes (Kerem *et al.*, J. Biol. Chem. 268, 180-184, 1993) and anchor AChE to their surfaces. The third splicing option involves continuous transcription through pseudointron I4, to yield the E1-E2-E3-E4-I4-E5 mRNA transcript. This transcript has been detected in embryonic and tumor cells (AChE-R, the so called "readthrough" form), as well as in stressed brain. Translation of AChE-R mRNA results in a 26 residue hydrophilic C-terminal extension devoid of cysteine (Li *et al.*, J. Biol. Chem. 266, 23083-90, 1991). In human AChE-transgenic *Xenopus* tadpoles, this isoform is secreted largely as soluble monomers, unlike the AChE-S isoform which accumulates in synapses.

All three variants share the same 534 residue core domain. When recombinant AChE-R and AChE-S were produced in micro- injected *Xenopus* oocytes and subjected to immunoblot analysis, a certain degree of natural C-terminal truncation occurred which created fast-migrating bands, with a molecular weight similar to that of the core protein, i.e. without the C-terminus, in both preparations (Sternfeld *et al.*, J. Neurosci. 18, 1240-9, 1998).

It has now surprisingly been found that a peptide derived from Acetylcholinesterase has independent biological activities. The invention is directed to a peptide comprising sequence derived from the open reading frame of an acetylcholinesterase mRNA splice variant. In a preferred

embodiment of the invention, the splice variant is AChE-R or AChE-S. The peptide of the invention comprises preferably from 9 to 100 amino acids, of the AChE mRNA open reading frame. More preferably, the peptide of the invention is derived from said AChE mRNA open reading frame.

The peptide of the invention preferably comprises a major region predicted to be rich in turns and β-pleated sheets. Also preferably, the peptide of the invention comprises an amphipathic helix structure, preferably unilaterally hydrophobic, which is preferably of a length of about 10 to about 30, more preferably abut 17 amino acid residues. Further preferably, the peptide of the invention is of low immunogenicity. This may be tested by injecting the peptide to an experimental animal, in the presence of adjuvants as known in the art. A peptide of low immunogenicity will not elicit antibodies unless conjugated or otherwise modified. Further details of antibody generation are disclosed hereinbelow, e.g., in the Experimental Procedures section.

In a preferred embodiment of the invention, the peptide comprises AChE sequence derived from the C-terminus thereof. The peptide sequence is preferably derived from between 9 to 100 amino acids from the C-terminus of the AChE translation product. More preferably, the peptide sequence is derived from the C-terminus, preferably from between 9 to about 100 amino acids of the C-terminus, of the mature AChE protein.

Preferably, the peptide comprises amino acid sequence corresponding to ACHE intron 4, exon 4, exon 5, and/or exon 6 sequence. More preferably, the peptide comprises intron 4 and exon 5 sequence, or

exon 4 and exon 6 sequence, or exon 5 and exon 6 sequence. Thus, in a preferred embodiment of the invention, the peptide is derived from the AChE-R or AChE-S mRNA open reading frames. Preferably, the peptide is derived from the C-terminus of the protein coded by said open reading frames, and more preferably, the peptide is derived from the C-terminus of the mature protein coded by said AChE-R or AChE-S mRNAs. In a preferred embodiment of the invention, the peptide comprises about 50, more preferably about 30, and most preferably about 26 amino acids of the mature C-terminus of AChE-R or AChE-S encoded protein.

The peptide of the invention comprises more preferably between 20-50 amino acids of the C-terminus of a mature AChE protein, which is preferably the protein encoded by the AChE-R or AChE-S mRNAs.

In a more preferred embodiment, the peptide comprises the C-terminal 26 amino acids of the mature translation product of AChE-R mRNA, which is the amino acid sequence N-terminus GMQGPAGSGWEEGSGSPPGVTPLFSP C-terminus, also denoted herein as SEQ ID No. 1. A peptide having said 26 C-terminal amino acids is denoted herein as ARP, which is the most preferred embodiment of the invention.

In another preferred embodiment, the peptide of the invention comprises the C-terminus between about 30 and about 50 amino acids of the protein encoded by the AChE-S mRNA. More preferably, the peptide comprises about 40 amino acids of the mature C-terminus of the protein encoded by the AChE-S mRNA. The peptide having 40 amino acids derived

from the C-terminus of the protein encoded by the AChE-S mRNA is denoted herein as ASP, or SEQ. ID. No. 2. Another preferred embodiment is the non-helical region of SEQ ID No. 2, i.e., the 27 C-terminal amino acids thereof, also denoted herein as SEQ. ID No. 3.

The peptide of the invention may comprise sequences derived from sources other than the above-described, so long as the function of said peptide is not substantially affected. The additional sequences are generally zero to 300 amino acids in length, and may comprise sequences derived from acetylcholinesterase, growth factors, enzyme, structural proteins, or non-natural sequences.

Acetylcholinesterase sequences that may be added may e.g., be taken from the above-described exons 4, 5, and/or 6, and/or intron 4 sequences, for the purposes of creating an internally linked peptide dimer. Dimers may possess enhanced activity, see e.g., Corcoran *et al.*, Eur. Cytokine Netw. 9, 255-62, 1998. Dimer formation of the peptide of the invention may be achieved e.g., by inclusion of cysteines in the peptide sequence. This may be achieved either by choosing cysteines in the natural acetylcholinesterase-derived sequence of the peptide, or by adding cysteine residues to the peptide sequence, or by adding to the peptide sequence such amino acid sequences that contain cysteine residues, preferably amino acid sequences which are known to form dimers.

Dimerization may also be achieved by fusing the desired peptide sequence to an IgG backbone. This technique is described e.g., in the above Corcoran *et al.*

The peptide of the invention may comprise also growth factor sequences. This may be useful e.g., when it is desired to enhance survival and growth of hematopoietic stem cells. The growth factors are in that case preferably selected from early-acting factors, such as IL3, IL6, TPO and/or FLT3. When it is desired to enhance expansion of hematopoietic stem cells, the amino acid sequence of growth factors such as GM-CSF or preferably SCF, may be added to the peptide sequence.

Where known, the active peptide of such growth factors may be used instead of the entire growth factor sequence. Of course, dimerization of the peptide of the invention may also enhance growth factor activity and/or binding affinity.

In some cases, it may be desired to promote differentiation of a cell derived from a hematopoietic source. Such differentiation may be especially desired if said cell has acquired growth-factor independent uncontrolled growth characteristics. The peptide of the invention may in such cases be fused to a toxin, which upon entering the cell, may exert cytostatic effects, in addition to the differentiation-promoting effect of the peptide of the invention. Fusion of peptides to toxins has been described in many research and review articles, see e.g., Pastan *et al.*, Science 254:1173-7, 1991.

One of the effects of the peptide of the invention is targeted to bone marrow stem cells. Therefore, when it is desired to provide the peptide of the invention for use in treating a patient in need of such treatment, it may be desirable to target the peptide of the invention to the cells desired.

This may be achieved by fusing the peptide to a sequence capable of binding to a bone marrow stem cell surface marker. One example for such a marker is the CD34 antigen. Consequently, the peptide of the invention may comprise CD34-ligand sequence. Alternatively, the peptide of the invention may comprise anti-CD34 single chain sequences. The preparation of antibodies and single-chain antibodies is well known in the art, see also below.

When it is desired to add amino acid sequences or domains to the peptide of the invention, it may be desired to separate the acetylcholinesterase-derived part of the sequences from the additional sequences by way of a linker sequence. Linker sequences may consist mainly of amino acids that do not provide spatial constraints, such as glycine and preferably alanine. An example for a flexible peptide linker sequence is described in e.g., White *et al.*, J. Immunol. 162, 2671-6, 1999.

The peptide of the invention comprises most preferably SEQ ID No. 1 or 2. However, it is to be understood that the invention pertains to any peptide comprising sequence structurally similar to AChE sequence with substantially equal or greater activity. Changes in the structure of the peptide comprise one or more deletions, additions, or substitutions. The number of deletions or additions, which may occur at any point in the sequence, including within the acetylcholinesterase-derived sequence, will generally be less than 25%, preferably less than 10% of the total amino acid number. This figure does not include additions as described above, e.g., addition of sequences coding for growth factor sequences.

Preferred substitutions are changes that would not be expected to alter the secondary structure of the peptide, i.e., conservative changes. The following list shows amino acids that may be exchanged (left side) for the original amino acids (right side).

Original Residue	Exemplary Substitution
Ala	Gly;Ser
Arg	Lys
Asn	Gln;His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala; Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Tyr; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr

Tyr	Trp; Phe
Val	Ile; Leu

Amino acids can also be grouped according to their essential features, such as charge, size of the side chain, and the like. The following list shows groups of similar amino acids. Preferred substitutions would exchange an amino acid present in one group with an amino acid from the same group.

1. Small aliphatic, nonpolar: Ala, Ser, Thr Pro, Gly;
2. Polar negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar positively charged residues: His, Arg, Lys;
4. Large aliphatic nonpolar residues: Met, Leu, Ile, Val, Cys;
5. Large aromatic residues: Phe, Tyr, Trp.

Further comments on amino acid substitutions and protein structure may be found in Schulz *et al.*, Principles of Protein Structure, Springer-Verlag, New York, NY, 1798, and Creighton, *T.E.*, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, CA 1983.

The preferred conservative amino acid substitutions as detailed above are expected to substantially maintain or increase the function or activity of the peptide of the invention, as detailed hereinbelow. Of course,

any amino acid substitutions, additions, or deletions are considered to be within the scope of the invention where the resulting peptide is a peptide of the invention, i.e., a peptide which is substantially equal or superior in terms of function to the preferred peptide of the invention.

The peptide of the invention may be further modified to improve its function, affinity ,or stability. For instance, cyclization may be used to impart greater stability and/or overall improved performance upon the peptide. A number of different cyclization methods have been developed, including side chain cyclization and backbone cyclization. These methods are well documented in the prior art, see e.g., Yu *et al.*, Bioorg. Med. Chem. 7, 161-75, 1999, Patel *et al.*, J. Pept. Res. 53, 68-74, 1999, Valero *et al.*, J. Pept. Res. 53, 56-67, 1999, Romanovskis *et al.*, J. Pept. Res. 52, 356-74, 1998, Crozet *et al.* Mol. Divers. 3, 261-76, 1998, Rivier *et al.*, J. Med. Chem. 41, 5012-9, 1998, Panzone *et al.*, J. Antibiot. (Tokyo), 51, 872-9, 1998, Giblin *et al.*, Proc. Natl. Acad. Sci. USA 95, 12814-8, 1998, Limal *et al.*, J. Pept. Res. 52:121-9, 1998, and US Patent (USP) 5,444,150.

A preferred method of cyclization involves stabilization of an amphipathic alpha-helix by using para-substituted amino acid derivatives of a benzene ring, see the above Yu *et al.*, Bioorg. Med. Chem. 7:161-75, 1999. Another preferred method of cyclization is backbone cyclization, as disclosed in Reissmann *et al.*, Biomed. Pept. Proteins Nucleic Acids 1:51-6, 1994-95, and in references therein. A relatively new method of cyclization which involves backbone-to side chain connections may also be used (see the above Reissmann *et al.*).

Other modifications as known in the art may be carried out. For instance, it may be desirable to link polyethylenglycol (PEG) groups to the peptide. Such groups may impart enhanced stability upon the peptide. Another effect of these groups may be lowered immunogenicity. This feature of PEG-linked peptides may be particularly desirable when the peptide of the invention is to be used *in vivo*. Preparation of PEG-linked peptides has been described by Guerra *et al.*, Pharm. Res. 15:1822-7, 1998.

The present invention provides AChE-derived peptides. A therapeutic or research-associated use of these tools may necessitate their introduction into or interaction with tissue cultured cells or cells of a living organism. For this purpose, or for assisting in the penetrance of such peptides into the brain through the blood-brain barrier, it is desired to improve membrane permeability of the peptides. The principle of derivatization with lipophilic structures may be used in creating peptides with enhanced membrane permeability. For instance, the sequence of a known membranotropic peptide (see e.g., Soukchareun *et al.*, Bioconjug. Chem. 9, 466-75, 1998) may be added to the sequence of the peptide of the invention. Further, the peptide may be derivatized by partly lipophilic structures such as Palmityl or Geraniol groups. For instance, lauroyl derivatives of peptides have been described by Muranishi *et al.*, Pharm. Research 8, 649, 1991. Further modifications of peptides comprise the oxidation of methionine residues to thereby create sulfoxide groups, as described by Zacharia *et al.*, Eur. J. Pharmacol. 203, p. 353, 1991. Zacharia and coworkers also describe peptide or derivatives wherein the relatively

hydrophobic peptide bond is replaced by its ketomethylene isoester (COCH2). These and other modifications known to the person of skill in the art of protein and peptide chemistry enhance membrane permeability.

Another way of enhancing membrane permeability is the use of receptors, such as virus receptors, on cell surfaces in order to induce cellular uptake of the peptide or protein. This mechanism is used frequently by viruses, which bind specifically to certain cell surface molecules. Upon binding, the cell takes the virus up into its interior. The cell surface molecule is called a virus receptor. For instance, the integrin molecules CAR and AdV have been described as virus receptors for Adenovirus, see Hemmi *et al.*, Hum. Gene Ther. 9, 2363-73, 1998, and references therein. The CD4, GPR1, GPR15, and STRL33 molecules have been identified as receptors/coreceptors for HIV, see Edinger *et al.* Virology 249, 367-78, 1998 and references therein.

Thus, conjugating peptides, proteins or oligonucleotides to molecules that are known to bind to cell surface receptors will enhance membrane permeability of said peptides, proteins or oligonucleotides. Examples for suitable groups for forming conjugates are sugars, vitamins, hormones, cytokines, transferrin, asialoglycoprotein, and the like molecules. Low *et al.*, USP 5,108,921, describe the use of these molecules for the purpose of enhancing membrane permeability of peptides, proteins and oligonucleotides, and the preparation of said conjugates. Of course, as one type of the cells targeted by the peptide of the invention are hematopoietic stem cells, it is advantageous to chose a cell surface protein that will occur

preferably on such cells, such as a hematopoietic stem cell surface marker, preferably the CD34 molecule.

Alternatively, membrane permeability may be enhanced targeting the peptide to ubiquitous cell surface structures. Low and coworkers (see above) teach that molecules such as folate or biotin may be used to target a conjugate molecule to a multitude of cells in an organism, because of the abundant and unspecific expression of the receptors for these molecules.

The above use of cell surface proteins for enhancing membrane permeability of a peptide of the invention may also be used in targeting said peptide of the invention to certain cell types or tissues. For instance, if it is desired to target cancer cells, it is preferable to use a cell surface protein that is expressed more abundantly on the surface of those cells. Examples are the folate receptor, the mucin antigens MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC7, the glycoprotein antigens KSA, carcinoembryonic antigen, prostate-specific membrane antigen (PSMA), HER-2/neu, and human chorionic gonadotropin-beta. The above-noted Wang *et al.*, 1998, teaches the use of folate to target cancer cells, and Zhang *et al.*, Clin. Cancer Res. 4, 2669-76 1998, teaches the relative abundance of each of the other antigens noted above in various types of cancer and in normal cells. As the peptide of the invention preferably acts to promote differentiation of hematopoietic stem cells, other markers may be used, as advantageous in each particular case. The above-noted CD34 antigen, or the CD41 and CD33 antigens, may be used

as useful markers for targeting a peptide of the invention to uncontrollably growing cells that are derived from hematopoietic stem cells.

The protein, peptide or oligonucleotide of the invention may therefore, using the above-described conjugation techniques, be targeted to certain cell type as desired. For instance, if it is desired to enhance differentiation in cells of the lymphocytic lineage, a peptide of the invention may be targeted at such cells, for instance, by using the MHC class II molecules that are expressed on these cells. This may be achieved by coupling an antibody, or the antigen-binding site thereof, directed against the constant region of said MHC class II molecule to the protein or peptide of the invention. Further, numerous cell surface receptors for various cytokines and other cell communication molecules have been described, and many of these molecules are expressed in more or less tissue- or cell-type restricted fashion. Thus, for instance, when it is desired to target a subgroup of T cells, the CD4 T cell surface molecule may be used for producing the conjugate of the invention. CD4-binding molecules are provided by the HIV virus, whose surface antigen gp42 is capable of specifically binding to the CD4 molecule.

The peptides of the invention may be introduced into cells by the use of a viral vector. The use of vaccinia vector for this purpose is detailed in chapter 16 of the above-noted Current Protocols in Molecular Biology. The use of Adenovirus vectors has been described e.g. by Teoh *et al.*, Blood 92, 4591-4601, 1998, Narumi *et al.*, Am. J. Respir. Cell. Mol. Biol. 19, 936-941, 1998, Pederson *et al.*, J. Gastrointest. Surg. 2, 283-91, 1998, Guang-Lin *et*

al., Transplant. Proc. 30, 2923-4, 1998, and references therein, Nishida *et al.*, Spine 23, 2437-42, 1998, Schwarzenberger *et al.*, J. Immunol. 161, 6383-9, 1998, and Cao *et al.*, J. Immunol. 161, 6238-44, 1998. Retroviral transfer of antisense sequences has been described by Daniel *et al.*, J. Biomed. Sci. 5, 383-94, 1998.

When using viruses as vectors, the viral surface proteins are generally used to target the virus. As many viruses, such as the above Adenovirus, are rather unspecific in their cellular tropism, it may be desirable to impart further specificity by using a cell-type or tissue-specific promoter. Griscelli *et al.*, Hum. Gene Ther. 9, 1919-28, 1998 teach the use of the ventricle-specific cardiac myosin light chain 2 promoter for heart-specific targeting of a gene whose transfer is mediated by Adenovirus. The peptide of the invention is preferably targeted to hematopoietic progenitor cells. Promoters and transcription factor binding motifs involved in hematopoietic-specific transcription have been described in a number of articles. It is also possible to isolate and use in the practice of the invention a promoter of a known gene which is specifically expressed in hematopoietic stem cells, such as the SZF1 gene (Liu *et al.*, Exp. Hematol. 27, 313-25, 1999), or the CD34 gene (see US 5,556,954). Further examples of transcription factor binding motifs and promoters involved in hematopoietic stem cell specific transcription may be found e.g., in Onyango *et al.*, Exp. Hematol. 27, 313-25, 1999, Meng *et al.*, Blood 93, 500-8, 1999, Nony *et al.*, J. Biol. Chem. 273, 32910-9, 1998, and Ye *et al.*, Hum. Gene Ther. 9, 2197-205, 1998.

Isolation of 5' gene sequences and of the promoter contained therein may be carried out by routine procedures, e.g., using cosmid or P1 phage libraries, hybridization using cDNA sequences, and isolation of genomic 5' gene fragments and testing same for promoter activity, e.g., using the Chloramphenicolacetyltransferase or luciferase genes as reporter genes. Such techniques are detailed e.g., in Current Protocols in Molecular Biology, Ausubel *et al.* (eds.), John Wiley and Sons, New York, NY, and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, see also Jiang *et al.*, J. Biol. Chem. 274, 7893-900, 1999, Lennon *et al.*, Immunogenetics 45, 266-73, (1997), Grombacher *et al.*, DNA Cell. Biol. 15, 581-8 (1996), Scherer *et al.*, Hum. Genet. 97, 114-6, 1996, Dirks *et al.*, J. Interferon Res. 9, 125-33 (1989).

Alternatively, or in addition, the viral vector may be engineered to express an additional protein on its surface, or the surface protein of the viral vector may be changed to incorporate a desired peptide sequence. The viral vector may thus be engineered to express one or more additional epitopes which may be used to target said viral vector. For instance, cytokine epitopes, MHC class II-, CD34-, CD33-, CD41-binding peptides, or epitopes derived from homing molecules, may be used to target the viral vector in accordance with the teaching of the invention.

The peptide of the invention possesses one or more of the following activities:

-stem cell survival promoting activity;

-stem cell expansion promoting activity;

-stem cell-derived cell differentiation promoting activity;

-AChE mRNA inducing activity in stem cells;

-SCF substitution activity for hematopoietic stem cells;

-rescue of GM-CSF activity in hematopoietic stem cells in the presence of anti-AChE antisense oligonucleotides;

-induction of DNA synthesis activity in GM-CSF treated hematopoietic stem cells;

-enhancement of growth factor activity of GM-CSF, SCF and TPO.

The activity of the peptide according to the invention may be investigated by assays involving stem cell survival and growth. Such assays, which are known to the person of skill in the art, may be conducted either *in vivo* or *in vitro*. The term "stem cells" as used herein comprises stem cells of various origins and potency. It includes the totipotent embryonic stem cells, but also nerve, epithelial, and mesenchymal stem cells.

Preferred stem cells are embryonic stem cells, nerve stem cells, epithelial stem cells, mesenchymal stem cells, and hematopoietic stem cells. Particularly preferred embodiments of assays for the activities of the peptide of the invention are given in the Examples section hereinunder. It is to be understood that the examples, which refer to hematopoietic stem cells, may be carried out in a similar manner, following identical principles of method and analysis, with other stem cells, as mentioned above.

Embryonic stem cells may be obtained from totipotent cells of an embryo, according to procedures known in the art. A number of

publications describes embryonic stem cells of various origins and their obtention (Robertson E., in: Teratocarcinoma and Embryonic stem cells: A practical Approach, Robertson, E. (ed.), IRL Press, Oxford, p. 71, 1987, Dushnik-Levinson and Benvenisty, Biol. Neonate 67, p. 77, 1995, Thomson *et al.*, Science 282, p. 1145, 1998). *In vitro* aggregation of embryonic stem cells may result in the formation of embryoid bodies. The cells of embryoid bodies are regionally partially differentiated, including cells of mesoderm, ectoderm, and endoderm lineages. The growth and differentiation of embryonic stem cells, or cells derived from embryoid bodies, or the formation of embryoid bodies, are processes that may be influenced by a peptide of the invention. The preferred influence of a peptide of the invention is stimulation of growth and/or differentiation of an embryonic stem cell.

Nerve stem cells may be obtained and characterized as known in the art, see e.g., Morrison *et al.*, Cell 96, 737-49, 1999 and references therein. Epithelial stem cells may be obtained and characterized as known in the art, see e.g., Cotsarelis *et al.*, Exp. Dermatol. 8, 80-8, 1999, and references therein. Mesenchymal stem cells may be obtained and characterized as known in the art, see e.g., Pittenger *et al.*, Science 284, 143-7, 1999, Horwitz *et al.*, Nat. Med., 5, 309-13, 1999, Ghilzon *et al.*, Leuk. Lymphoma 32, 211-21, 1999, Bruder *et al.*, Clin Orthop. 355 Suppl, S247-56, 1998, and references therein.

Hematopoietic stem cells may be prepared from sources such as bone marrow or umbilical chord blood. The procedures for obtaining such cells

have been described in a number of publications known to those of skill in the art, see e.g., US Patents 5,610,056, 5,728,581, 5,668,104, 5,199,942, and Ahmed *et al.*, Stem Cells 17:92-9, 1999. Crude stem cell cultures are often contaminated with non-pluripotent cells. It is therefore preferred to purify these cells, using a stem cell marker. Any stem cell marker, preferably a stem cell surface marker, as known in the art may be used. The preferred stem cell marker is CD34. Cells are purified using an agent capable of specifically binding the stem cell marker. Such agents may be e.g., ligands, synthetic compounds including peptides, or antibodies. A preferred agent is a specific anti-stem cell marker antibody. A preferred antibody is the anti-CD34 antibody. While antibodies may be prepared by methods known well to those of skill in the art (see below), a preferred anti-CD34 antibody is the CD34-PE, available from Becton Dickinson, Immunocytometry System Inc., Mountain View, Calif., USA. Stem cell marker enriched cells may be purified in a number of ways. For instance, the stem cell marker carrying cells may be labeled, using an antibody directed against the stem cell marker. The antibody is either labeled itself or is reacted with a second, labeled antibody. Examples of antibodies that may be used are the above CD34-PE (Phycoerythrin-labeled), or a combination of anti-CD34 (e.g., the said CD34-PE) and FITC-RaMIg. FITC-RaMIg are Fluorescein- isothiocyanate-labeled rabbit anti-mouse IgG antibodies. They should of course be used only if the first antibody is a mouse (usually monoclonal) antibody. FITC-RaMIg or similar labeled antibodies with the desired specificities are available from several

companies, including Sigma, St. Lois, Mo., USA, and PIERCE, Rockford, IL61105, USA.

Using the above stem cell surface marker binding agent, CD34+ cells may be purified in a variety of ways, including panning, fluorescence-activated cell sorting, or by using magnetic beads. Panning involves adsorbing cells binding a certain antibody to a surface covered with this antibody, and thus enriching these cells. See e.g., Hoogenboom *et al.*, Eur. J. Biochem. 260, 774-784, 1999, and references therein. The use of magnetic beads involves magnetic beads that carry the antibody. After the cells are bound to the bead via the antibody, a magnetic force is applied to separate the beads from the rest of the culture solution, thereby enriching the bound cells. Magnetic beads are commercially available, e.g., from Dynal, Norway. Antibodies and other agents may be bound to bead by a variety of techniques known to the person of skill in the art, e.g., via chemical cross-linking. Cross-linking agents and references regarding the procedure of cross-linking are disclosed, e.g., in the Life Science Products catalog of the above PIERCE.

Hematopoietic stem cells are then cultured as known in the art, see e.g., Current Protocols in Immunology is published by John Wiley & Sons. The concentration of the cells is kept initially low, i.e., at about 50,000 to about 250,000 cells per ml. A preferred medium is IMDM (see e.g., Bruserud *et al.*, J. Hematother. 8, 63-73, 1999). The medium preferably comprises autologous plasma, in an amount of between 5 and 30 %, preferably 10%. Other additions to the culture media are as known in the

art, preferably 2mM L-glutamine, 20IU/ml Heparin, and antibiotics Penicillin, Streptomycin, and Amphotericin B. Penicillin and Streptomycin are added to a final concentration of preferably 5μg to 2mg per ml, more preferably 100 μg/ml. Amphotericin B is added at a final concentration of 1 to 100 μM, preferably 20 μM.

To the culture of hematopoietic stem cells may be added early growth factors, inlcuding IL-3, IL-6, either alone or in combination with soluble IL-6 receptor, Thrombopoietin, stem cell factor, granulocyte-macrophage colony-stimulating factor, and FLT-3, or combinations thereof. The preferred concentrations of these agents may be inferred from the example section herein below, or alternatively, from the above US Patents 5,610,056, 5,728,581, 5,668,104, 5,199,942, and Ahmed *et al.*, Stem Cells 17:92-9, 1999.

A peptide according to the invention is added to the stem cell cultures, together with, or at different times than the above growth factors. The preferred peptide concentration is about 1 ng/ml to about 1 500 ng/ml, more preferred about 50 to about 100 ng/ml.

Growth factors and the peptide according to the invention may be supplemented preferably every 24 hours to about every ten days, more preferably every 3-5 days. The cultures are grown for about 14 hours to about three months, more preferably for about 24 hours to about one month.

Cultures of stem cells may then be analyzed by cytochemical staining, by cell proliferation assay, viable cell count, cell phenotyping, for

CD34, CD33, and CD 41 markers, and growth of progenitor colonies, using established techniques, preferably as described hereinbelow. Cell survival may also be evaluated by determining dead cell counts, e.g., using apoptosis-specific reagents. Such reagents in kit form are available from many companies, including Clontech Laboratories UK Ltd., Hampshire RG24 8NE, UK, InterGen Energy Inc., Boston, MA 02114, USA, R&D Systems, Minneapolis, MN 55413, USA, and Boehringer Mannheim/ Hoffmann-La Roche Ltd, 4070 Basel, Switzerland.

Stem cell growth may be quantified in a variety of ways, such as viable cell count using trypan blue exclusion (see hereinbelow), or measuring DNA replication. A large number of reagents and systems are commercially available for the purposes of quantifying DNA replication. For instance, incorporation of the nucleotide analog Bromo-deoxy-Uridine (BrdU) may serve as an indicator of DNA replication (see hereinbelow). However, also radioactive nucleotide analogs, such as ^{3}H-Thymidine, may be added to the culture medium and their incorporation into DNA measured by liquid scintillation counting. Techniques for measuring DNA replication are well known in the art and are described in many textbooks and articles.

Peptides of the invention, when used either alone or in combination with the above early cytokines, will promote stem cell survival significantly. Preferably stem cell survival is promoted by about 10%, more preferably by about 100%, and still more preferably by about 1000 or more

percent, when compared to control cultures lacking the peptide of the invention.

It is understood by the skilled person that promotion of stem cell survival by a peptide of the invention will usually be highest in terms of percentage in the absence of other growth factors. Further, the percentage of growth promotion by the peptide of the invention depends upon the growth factor(s) used in combination with the peptide of the invention, on the culture conditions, and on the source and type of stem cells used for the assay.

In another assay, the peptide of the invention may be tested for its ability to promote stem cell differentiation. For instance, hematopoietic stem cells expanded by growth in a medium comprising a peptide of the invention will display increased ability to differentiate into megakaryocytic (MK) and myeloid cells. The differentiation of cells may be assayed from about three days in culture to about three months, preferably from about one week in culture to about three weeks, and more preferably at about two weeks in culture medium comprising a peptide of the invention. The differentiated morphology of the cells may be analyzed microscopically, including analysis by light microscopy, electron microscopy. Alternatively, the differentiation of stem cells may conveniently be analyzed using cell surface differentiation markers, such as cluster of differentiation (CD) markers. A preferred CD marker for myeloid differentiation of hematopoietic stem cells is the CD33 marker. A preferred marker for megakaryocytic differentiation is the CD41 marker. Analysis of these

markers may be carried out as known in the art, e.g., using antibodies specific for the desired marker, either labeled or in conjunction with labeled second antibodies. The label may then be detected by enzymatic reaction, fluorescence microscopy, fluorescence-activated cell sorting, or the like techniques.

The activity of the peptide of the invention upon differentiation of stem cells in culture may thus be determined by the analysis of differentiation-associated markers. A peptide of the invention having differentiation-promoting activity will significantly enhance the number of cells in a culture that express morphology of differentiated cells, or that express a differentiation marker. The enhancement (as compared to control cultures lacking the peptide of the invention) is preferably at least about 30%, more preferably at least 150%, still more preferably between at least 100 and at least 1000%.

The AChE production inducing activity of the peptide invention may be tested by culturing stem cells or stem cell-derived cells in the presence and in the absence of the peptide of the invention. The amount of AChE in these cells may then be quantified by techniques known in the art, such as immunochemical or cytochemical staining, AChE enzyme assay, and the like. For detection by immunochemical staining, anti-AChE antibodies may be used; however, also AChE-specific substrates can be employed (see hereinbelow). The addition of AChE-specific enzyme inhibitors such as iso-OMPA (see e.g., Keymer *et al.*, Eur. J. Neurosci. 11, 1049-57, 1999) in this assay may serve as a control reaction.

The above-detailed activities of the peptide of the invention make it useful in the treatment of various disorders or conditions in which growth enhancement of stem cells is desired. In a preferred embodiment, the condition is thrombocytopenia or a post-irradiation condition, or a chemotherapy condition, or a condition of massive blood loss.

The invention also comprises anti-AChE antibodies and the use thereof for the diagnosis of pathological conditions, particularly hematopoietic pathological conditions. Polyclonal antibodies may be generated in rabbits, chicken, mice, rats, sheep, or similar mammals. For generation of antibodies against a peptide of the invention, the peptide is produced by recombinant DNA technology in mammalian cells, as described in the above general references for molecular biology. Alternatively, the peptide may be synthetically produced by organic chemistry. The peptide may also be produced in bacterial or insect cells as detailed in the above-noted Current Protocols in Molecular Biology, chapter 16.

The peptide is purified from the cells in which it has been produced. Peptide purification methods are known to the person of skill in the art and are detailed e.g., in the above-noted Current Protocols in Molecular Biology, chapter 16, and in Current Protocols in Protein Science, Wiley and Sons Inc. Chapters 5 and 6. Advantageously, the peptide may be produced as a fusion with a second protein, such as Glutathione-S-transferase or the like, or a sequence tag, such as the histidine tag sequence. The use of fusion or tagged proteins simplifies the purification procedure, as detailed

in the above-noted Current Protocols in Molecular Biology, chapter 16, and in the instructions for the his-tag protein expression and purification kit, as available from Qiagen GmbH, 40724 Hilden, Germany.

If the protein or peptide has been expressed as a fusion protein, it may be desirable to cleave the fusion partner before using the protein for the generation of antibodies, in order to avoid generation of antibodies against the fusion partner. The cleavage of fusion partners and the isolation of the desired protein is described in the above-noted Current Protocols in molecular Biology, chapter 16. Vectors, protocols and reagents for expressing and purifying maltose-binding protein fused recombinant proteins are also available commercially.

When producing a peptide of the invention, it may be desirable not to remove the fusion partner, as the fusion protein may stimulate the production of antibodies against the peptide. Generally, this consideration may be relevant when generating antibodies from peptides that are less than 50 amino acids in length. In particular, it has been found that the ARP peptide, when injected, is virtually non-immunogenic. A Keyhole Limpet hemocyanin (KLH)-conjugated ARP peptide was found to elicit antibodies unable to detect ARP or acetylcholinesterase. Antibodies capable of detecting ARP were successfully generated using a Glutathione-S-transferase-ARP fusion protein (detailed hereinbelow). Accordingly, in a preferred embodiment of the invention, antibodies are elicited using a conjugate or fusion protein of the peptide of the invention as antigen. A preferred fusion partner is Glutathione-S-transferase.

As noted further above, the peptide may also be synthesized by chemical methods known in the art of chemistry.

The generation of polyclonal antibodies against proteins is described in chapter 2 of Current Protocols in Immunology, Wiley and Sons Inc. The generation of antibodies against peptides may necessitate some changes in protocol, because of the generally lower antigenicity of peptides when compared to proteins. The generation of polyclonal antibodies against peptides is described in the above-noted Current Protocols in Immunology, chapter 9, and exemplified hereinbelow.

Monoclonal antibodies may be prepared from B cells taken from the spleen or lymphnodes of immunized animals, in particular rats or mice, by fusion with immortalized B cells under conditions which favor the growth of hybrid cells. For fusion of murine B cells, the cell line Ag-8 is preferred.

The technique of generating monoclonal antibodies is described in many articles and textbooks, such as the above-noted chapter 2 of Current Protocols in Immunology. Chapter 9 therein describes the immunization, with peptides, of animals. Spleen or lymphnode cells of these animals may be used in the same way as spleen or lymphnode cells of protein-immunized animals, for the generation of monoclonal antibodies as described in chapter 2 therein.

The techniques used in generating monoclonal antibodies are further described in Kohler and Milstein, Nature 256, 495-497, 1975 and in USP 4,376,110.

In the preparation of antibodies from a gene bank of human antibodies the hypervariable regions thereof are replaced by almost random sequences, is described in USP 5,840,479. This method of antibody generation is preferred if it is difficult to immunize an animal with a given peptide or protein. The peptide of the invention may be poorly immunogenic, even as a conjugate. The antibodies described in USP 5,840,479 are further preferred if it is desired to use antibodies with a structure similar to human antibodies, for instance, when antibodies are desired that have low immunogenicity in humans.

Once a suitable antibody has been identified, it may be desired to change the properties thereof. For instance, a chimeric antibody may achieve higher yields in production. Chimeric antibodies wherein the constant regions are replaced with constant regions of human antibodies are further desired when it is desired that the antibody be of low immunogenicity in humans. The generation of chimeric antibodies is described in a number of publications, such as Cabilly *et al.*, Proc. Natl. Acad. Sci. USA 81, 3273, 1984, Morrison *et al.*, Proc. Natl. Acad. Sci. USA 81, 6851, 1984, Boulianne *et al*, Nature 312, 643, 1984, EP 125023, EP 171496, EP 173494, EP 184187, WO 86/01533, WO 87/02671, and Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring harbor Laboratory, 1988.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and $F(ab')_2$, which are capable of binding antigen. Fab and $F(ab')_2$ fragments lack the Fc

fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl *et al.*, J. Nucl. Med. 24, 316-325, 1983).

It will be appreciated that Fab and $F(ab')_2$ and other fragments of the antibodies useful in the present invention may be used for the detection and quantitation of the peptide of the invention and of intact AChE or its isoforms, according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody, that can also be recognized by that antibody. Epitopes or "antigenic determinants" usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains, and have specific three-dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody, which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the

multitude of other antibodies which may be evoked by other antigens.

The antibodies, including fragments of antibodies, useful in the present invention, may be used to quantitatively or qualitatively detect the peptide of the invention, in a sample. This can be accomplished by immunofluorescence techniques employing a fluorescently or color-labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorometric detection.

The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of a peptide of the invention. *In situ* detection may be accomplished by removing a histological specimen from a mammal, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the peptide, but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Such assays for the peptide of the invention typically comprise incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells such as lymphocytes or leukocytes, or cells which have been incubated in tissue culture, in the presence of a labeled antibody

capable of identifying the peptide, and detecting the antibody by any of a number of techniques well known in the art.

The biological sample may be treated with a solid phase support or carrier such as nitrocellulose, or other solid support or carrier which is capable of immobilizing cells, cell particles or soluble proteins. The support or carrier may then be washed with suitable buffers followed by treatment with a detectably labeled antibody in accordance with the present invention, as noted above. The solid phase support or carrier may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support or carrier may then be detected by conventional means.

By "solid phase support", "solid phase carrier", "solid support", "solid carrier", "support" or "carrier" is intended any support or carrier capable of binding antigen or antibodies. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon amylases, natural and modified celluloses, polyacrylamides, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support or carrier configuration may be spherical, as in a bead, cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports or carriers include polystyrene beads. Those skilled in

the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of antibody, of the invention as noted above, may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

Other such steps as washing, stirring, shaking, filtering and the like may be added to the assays as is customary or necessary for the particular situation.

One of the ways in which an antibody in accordance with the present invention can be detectably labeled is by linking the same to an enzyme and used in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholin-esterase. The detection can be accomplished by colorimetric

methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may be accomplished using any of a variety of other immunoassays. For example, by radioactive labeling the antibodies or antibody fragments, it is possible to detect receptor tyrosine phosphatase (R-PTPase) through the use of a radioimmunoassay (RIA). A good description of RIA may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work, T.S. *et al.*, North Holland Publishing Company, NY (1978) with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, T., incorporated by reference herein. The radioactive isotope can be detected by such means as the use of a g counter or a scintillation counter or by autoradiography.

It is also possible to label an antibody in accordance with the present invention with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can be then detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrine, pycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}E, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups

as diethylenetriamine pentaacetic acid (ETPA).

The antibody can also be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

An antibody molecule of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support or carrier and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted

with the sample being tested to extract the antigen from the sample by formation of a binary solid phase antibody-antigen complex. After a suitable incubation period, the solid support or carrier is washed to remove the residue of the fluid sample, including unreacted antigen, if any, and then contacted with the solution containing an unknown quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the antigen bound to the solid support or carrier through the unlabeled antibody, the solid support or carrier is washed a second time to remove the unreacted labeled antibody.

In another type of "sandwich" assay, which may also be useful with the antigens of the present invention, the so-called "simultaneous" and "reverse" assays are used. A simultaneous assay involves a single incubation step as the antibody bound to the solid support or carrier and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support or carrier is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support or carrier is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support or carrier after a suitable incubation period is utilized. After a second incubation, the solid phase is washed in

conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support or carrier is then determined as in the "simultaneous" and "forward" assays.

The present invention provides an immunoassay for the detection and quantification of a peptide of the invention. The creation of immunoassays, such as RIA or ELISA, has been described in many articles, textbooks, and other publications. Reference is made to WO 97/03998, p. 48, line 4 to p. 52, line 27. Immunoassays of the invention may be if two general types: Firstly, immunoassays using an immobilized peptide of the invention, may be used. Secondly, immunoassays using immobilized antibodies directed against an epitope of a peptide of the invention may be used to quantify a peptide of the invention.

In a preferred embodiment of the invention, the assay is an immunoblot assay. The sample, e.g., a serum sample, is diluted, e.g., 1:10, in order to avoid overloading. The sample is then loaded onto a polyacrylamide gel, optionally a gradient gel, and electrophoresed. Synthetic or recombinantly produced peptide of the invention, preferably SEQ. ID. No. 1, SEQ ID No. 2, or SEQ ID No. 3, may be added in separate lanes or spiked to the sample lanes, as positive controls. The gel is then blotted, preferably onto a Nitrocellulose or Nylon membrane. The blot is reacted with antibodies against the peptide of the invention, preferably antibodies reactive with SEQ. ID No. 1, 2 or 3. A more preferred antibody is the rabbit anti-GST-ARP antibody as described herein. Bound antibody

may then be detected by antibodies reactive with the antibody of the invention, e.g., anti-rabbit immunoglobulins. These immunoglobulins are preferably labeled, e.g., by Peroxidase conjugation. The detection of the label is then carried out according to methods known in then art. Preferably, peroxidase-conjugated immunoglobulins are detected using the ECL™ detection system (Amersham Pharmacia Biotech, UK).

As described above, a preferred sample is serum. However, other body fluids may be used, including cerebrospinal fluid, liquor, saliva, and the like. Also, liquid extracts of body tissue may be analyzed. Alternatively, body tissue may be analyzed without extraction using cytochemical staining or immunostaining as described herein.

A preferred body fluid is cerebrospinal fluid. For instance, increased levels of AChE or of a peptide of the invention in cerebrospinal fluid, may be indicative of elevated blood cortisol levels, and may further be indicative of stress.

Such assays as hereinabove described may find use in diagnostics, as the level of the peptide of the invention may need to be evaluated in a number of conditions. For instance, such assays may be useful in order to monitor the effect of treatment of a patient with a peptide of the invention. Furthermore, such assays may be used in determining psychological stress (see Example 8 hereinbelow). Still further, such assays may be useful as an indicator of stress to the bone marrow. Stressed bone marrow will up-regulate ARP, which is a peptide of the invention, as detailed hereinunder in the Examples section. Finally, such assays may be useful in

the diagnosis of a number of disorders where growth or expansion of hematopoietic stem cells is adversely effected, or alternatively, uncontrolled growth of hematopoietic stem cells occurs.

Thus, in a preferred embodiment, the invention provides a method for the diagnosis of elevated glucocorticoid level; bone marrow stress, abnormality, dysfunction or stressed condition, or of increased platelet count or of brain infarct risk in a mammal, comprising obtaining a sample from said mammal, contacting said sample with an antibody of the invention, removing unbound antibody, and detecting the extent of reaction between said antibody and acetylcholinesterase or a fragment thereof present in said sample. The sample is preferably serum or a bone marrow sample.

A preferred embodiment of the invention is the use of a peptide of the invention in expansion of hematopoietic stem cells. This may be particularly useful in medical applications. As the amount of hematopoietic stem cells available for purposes such as transplantaion is typically very limited, there is a need for the expansion of hematopoietic progenitor cells for a number of clinical uses such as gene therapy, augmentation of bone marrow transplantation (BMT) and replacement of BMT. Such expansion may be either *ex-vivo* or *in-vivo*. US 5,861,315, and references therein, which are incorporated herein in their entirety by reference, describes methods for the expansion of hematopoietic stem cells, using a combination of the cytokines IL-6 and soluble IL-6 receptor. The combination of IL-6

and soluble IL-receptor is also said to sustain undifferentiated growth of embryonic stem cells.

Thus, in a preferred embodiment of the invention, a peptide of the invention is used for the *ex vivo* expansion of hematopoietic stem cells. The isolation, culture, expansion, and transplantation of such stem cell cultures has been described in many prior art articles, see e.g., the above US 5,861,315, Contassot *et al.*, Bone Marrow Transplant. 22, 1097-102, 1998, and Ahmed *et al.*, Stem Cells 17, 92-9, 1999.

In a preferred embodiment, mononuclear cells (MNC) obtained from human umbilical cord blood are separated by Ficoll-Hypaque density gradient centrifugation after depletion of phagocytes with Silica. $CD34^+$ cells are purified as described hereinabove and below, preferably using magnetic beads coated with anti-CD34 antibody. Purified $CD34^+$ cells are then incubated in suspension culture containing alpha-medium 20% fetal bovine serum 1% fraction V BSA. When cultured without serum, the culture preferably contains 2% pure BSA, 10 μg/ml insulin, 200 μg/ml transferrin, 10 μM beta-mercaptoethanol, and 40 μg/ml low-density lipoprotein instead of FBS and BSA. At regular time points, the distance of which is about three days to two weeks, the culture is diluted with fresh medium in an about 1:1 ratio. For assaying the number of hematopoietic progenitor cells, a sample is removed from the culture and the cells cultured in a clonal methylcellulose assay, e.g., as described by Nakahata *et al,* J. Clin. Invest., 70, 1324-1328, 1982, and in the above US 5,861,315. The culture contained alpha-medium, 0.9% methylcellulose, 30% FBS, 1%

BSA, 50 μM beta-mercaptoethanol, and cytokines. Methylcellulose cultures free of serum contain 1% BSA, 300 μg/ml human transferrin, 160 μg/ml soybean lecithin, and 96 μg/ml cholesterol instead of BSA and FBS. Cytokines to be added to the cultures include one or more of SCF, IL-1, IL-3, IL-6, soluble IL-6 receptor, IL-11, EPO, TPO, GM-CSF. Examples of preferred cytokines or combinations thereof are:

-IL-3+IL-6+TPO+FLT3;

-IL-6+sIL-6-R;

-IL-3+IL-6+sIL-6R+FLT3;

-IL-3+IL-11;

-IL-3+IL-11+TPO;

-IL-1+IL-3+TPO;

-SCF+IL-3+IL-6+TPO+FLT3;

-SCF+IL-3+IL-6+sIL-6R+FLT3;

-SCF+ IL-3+IL-11;

-SCF+IL-3+IL-11+TPO;

-SCF+IL-1+IL-3+TPO;

-SCF+IL-6+sIL-6-R.

Examples of preferred concentrations are e.g., IL3, 5 ng/mL, IL-6, 50 ng/mL, TPO 1 ng/mL, SCF, 10-100 ng/mL, FLT-3 ligand (FLT3), 50 ng/mL, GM-CSF, 50 ng/mL, sIL-6R, 1280 ng/ml, (see US 5,861,315, see also Ahmed *et al.*, Stem Cells 17, 92-9, 1999, see also Examples, particularly Example 3, and "Experimental procedures" section hereinbelow). The peptide of the invention is added to the cultures either concomitantly,

before, or after the cytokines, preferably in a concentration of about 50 ng/ml. However, it will be appreciated by the person of skill in the art that the optimal concentration of the peptide of the invention may vary according to its length, structure, and modification, and according to the combination of cytokines it is used with. Cytokines and the peptide of the invention are replenished as described hereinbelow, preferably about every 2 to 7 days, more preferably about every 4 days. The development of progenitors may be scored according to known criteria, see US 5,861,315, Koike *et al*, Exp. Med., 168, 879-890, 1988, Tanaka *et al*, Blood, 80, 1743-1749, 1992, Nakahata *et al*, J. Clin. Invest., 70, 1324-1328, 1982. The peptide of the invention preferably has megakaryocyte-differentiation promoting properties, as detailed in Example 3 hereinbelow. The activity of the peptide of the invention in promoting differentiation of stem cells into megakaryocytes is particularly advantageous when *ex-vivo* expanded stem cells are used for the purpose of bone marrow transplantation, or when transplanting such cells after a bone marrow transplantation. In these circumstances, thrombocytopenia is a frequent and sometimes fatal occurrence (see Ahmed *et al.*, Stem Cells 17, 92-9, 1999).

The peptide of the invention may be used in free form or as salt, e.g., as metal salt, including sodium, potassium, lithium or calcium salt, or as a salt with an organic base, or as a salt with a mineral acid, including sulfuric acid, hydrochloric acid or phosphoric acid, or with an organic acid e.g., acetic acid or maleic acid. Generally, any pharmaceutically acceptable salt of the peptide of the invention may be used.

The peptide of the invention may be used as such or in the form of a composition. A composition will generally contain salts, preferably in physiological concentration, such as PBS (phosphate-buffered saline), or sodium chloride (0.9% w/v), and a buffering agent, such as phosphate buffer in the above PBS. The preparation of pharmaceutical compositions is well known in the art, see e.g., US Patents 5,736,519, 5,733,877, 5,554,378, 5,439,688, 5,418,219, 5,354,900, 5,298,246, 5,164,372, 4,900,549, 4,755,383, 4,639,435, 4,457,917, and 4,064,236. The peptide of the present invention,. or a pharmacologically acceptable salt thereof is preferably mixed with an excipient, carrier, diluent, and optionally, a preservative or the like pharmacologically acceptable vehicles as known in the art, see e.g., the above US patents. Examples of excipients include, glucose, mannitol, inositol, sucrose, lactose, fructose, starch, corn starch, microcrystalline cellulose, hydroxypropylcellulose, hydroxypropyl- methylcellulose, polyvinylpyrrolidone and the like. Optionally, a thickener may be added, such as a natural gum, a cellulose derivative, an acrylic or vinyl polymer, or the like.

The pharmaceutical composition is provided in solid, liquid or semi-solid form. A solid preparation may be prepared by blending the above components to provide a powdery composition. Alternatively, the pharmaceutical composition is provided as lyophilized preparation. The liquid preparation is provided preferably as aqueous solution, aqueous suspension, oil suspension or microcapsule composition. A semi-solid composition is provided preferably as hydrous or oily gel or ointment.

About 0.001 to 60 w/v %, preferably about 0.05 to 25 w/v % of peptide is provided in the composition.

A solid composition may be prepared by mixing an excipient with a solution of the peptide of the invention, gradually adding a small quantity of water, and kneading the mixture. After drying, preferably *in vacuo*, the mixture is pulverized. A liquid composition may be prepared by dissolving, suspending or emulsifying the peptide of the invention in water, a buffer solution or the like. An oil suspension may be prepared by suspending or emulsifying the peptide of the invention or protein in an oleaginous base, such as sesame oil, olive oil, corn oil, soybean oil, cottonseed oil, peanut oil, lanolin, petroleum jelly, paraffin, Isopar, silicone oil, fatty acids of 6 to 30 carbon atoms or the corresponding glycerol or alcohol esters. Buffers inlcude Sorensen buffer (Ergeb. Physiol., 12, 393 1912), Clark-Lubs buffer (J. Bact., 2, (1), 109 and 191, 1917), Macllvaine buffer (J. Biol. Chem., 49, 183, 1921), Michaelis buffer (Die Wasserstoffinonenkonzentration, p. 186, 1914), and Kolthoff buffer (Biochem. Z., 179, 410, 1926).

A composition may be prepared as a hydrous gel, e.g. for transnasal administration. A hydrous gel base is dissolved or dispersed in aqueous solution containing a buffer, and the peptide of the invention, and the solution warmed or cooled to give a stable gel.

Preferably, the peptide of the invention is administered through intravenous, intramuscular or subcutaneous administration. Oral administration is expected to be less effective, because the peptide may be digested before being taken up. Of course, this consideration may apply

less to a peptide of the invention which is modified, e.g., by being cyclic peptide, by containing non-naturally occurring amino acids, such as D-amino acids, or other modification which enhance the resistance of the peptide to biodegradation. Decomposition in the digestive tract may be lessened by use of certain compositions, for instance, by confining the peptide of the invention in microcapsules such as liposomes. The pharmaceutical composition of the invention may also be administered to other mucous membranes. The pharmaceutical composition is then provided in the form of a suppository, nasal spray or sublingual tablet. The dosage of the peptide of the invention may depend upon the condition to be treated, the patient's age, bodyweight, and the route of administration, and will be determined by the attending physician. Doses ranging from 0.1 μg/kg to 100 mg/kg, preferably from 0.5 μg/kg to 5 mg/kg, more preferably 0.1μg/kg to 1mg/kg, most preferably about 100μg/kg.

The uptake of a peptide of the invention may be facilitated by a number of methods. For instance, a non-toxic derivative of the cholera toxin B subunit, or of the structurally related subunit B of the heal-labile enterotoxin of enterotoxic Eschericia coli may be added to the composition, see USP 5,554,378.

In another embodiment, the peptide of the invention is provided in a pharmaceutical composition comprising a biodegradable polymer selected from poly-1,4-butylene succinate, poly-2,3-butylene succinate, poly-1,4-butylene fumarate and poly-2,3-butylene succinate, incorporating

the peptide of the invention as the pamoate, tannate, stearate or palmitate thereof. Such compositions are described e.g., in USP 5,439,688.

In another embodiment, a composition of the invention is a fat emulsion. The fat emulsion may be prepared by adding to a fat or oil about 0.1-2.4 w/w of emulsifier such as a phospholipid, an emulsifying aid, a stabilizer, mixing mechanically, aided by heating and/or removing solvents, adding water and isotonic agent, and optionally, adjusting adding the pH agent, isotonic agent. The mixture is then homogenized. Preferably, such fat emulsions contain an electric charge adjusting agent, such as acidic phospholipids, fatty acids, bilic acids, and salts therof. Acidic phospholipids include phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid. Bilic acids include deoxycholic acid, and taurocholic acid. The preparation of such pharmaceutical compositions is described in US 5,733,877.

The invention will now be further described by way of the following non-limiting examples. Some general methods are described in the section entitled "experimental procedures" below, and are therefore not described in detail in the examples.

Experimental procedures

Cell source: UCB was collected, following informed consent of the parents and with the approval of the Sourasky Medical Center Ethics Committee, as previously described (Grisaru *et al.*, Am. J. Obstet. Gynecol, 180, 1240-1243, 1999). Following 1:1 (v/v) dilution in Iscove's modified

Dulbecco medium (IMDM, Beit Haemek, Israel), mononuclear cells were separated using 3% gelatin (Difco, Detroit, MI) and Ficoll-Hypaque gradients (<1.077 g/ml; Pharmacia, Uppsala, Sweden) (Pick *et al.*, Br. J. Haematol. 103, 639-50, 1998). $CD34^+$ cells were enriched using CD34 immunoglobulin-coated magnetic beads (CD34 progenitor cell selection system, Dynal, Norway). $CD34^+$ cells analysis was performed by flow cytometry (Becton Dickinson Immunocytochemistry System Inc., San Jose, CA), using CD34-PE (Becton Dickinson Immunocytometry System, Inc.) and CD45-FITC (Dako, Glostrup, Denmark) monoclonal antibodies. May-Grünwald- Giemsa staining revealed stem cell morphology.

Liquid cultures: UCB $CD34^+$ cells were set for liquid cultures at a concentration of 10^5/mL in IMDM, containing 10% autologous plasma, 2 mM L-glutamine (Sigma Chemical Co., St Louis, MO), penicillin (100 mg/mL), streptomycin (100 mg/mL), amphotericin B (2×10^{-5} M) (Sigma Chemical Co.), and heparin (20 IU/mL, Gibco, Grand Island, NY), all in a fully humidified atmosphere at 37°C and 5% CO_2. The following elements were added where noted:

1. Hematopoietic growth factors: Interleukin 3 (IL3, 5 ng/mL, Immunex, Seattle, WA), interleukin 6 (IL6, 50 ng/mL, R&D Systems, Minneapolis, MN), TPO (1 ng/mL, R&D Systems), stem cell factor (SCF, 10 ng/mL, R&D Systems), FLT-3 ligand (FLT3, 50 ng/mL, R&D Systems), granulocyte-macrophage colony stimulating factor (GM-CSF, 50 ng/mL, Biogenesis Ltd, Bournemouth, UK) and combinations of the above, for 24 hr to 28 days (supplemented every 4 days).

2. Stress mimicking conditions: Hydrocortisone sodium succinate (Abic Ltd., Netanya, Israel) at concentrations equivalent to normal, intermediate and stress serum cortisol levels (0.1, 0.6 and 1.2 μM, respectively (De Vroede *et al.*, Arch. Des. Child 78, 544-7, 1998), for 24 hr.

3. Antisense oligonucleotides: 3'-terminal 2'-O-Methylated 15- and 20-mer oligodeoxynucleotides in the antisense (AS) orientation, targeted against the common sequence domain in human AChEmRNA and BuChE mRNA, as control, were used for 24 hr, as detailed elsewhere (Grisaru *et al.*, Mol. Cell. Biol. 19, 788-95, 1999).

4. AChE C-terminal peptides: The following C-terminal peptides of the AChE synaptic (ASP, also denoted SEQ. ID. No. 2) and readthrough (ARP, also denoted SEQ ID. NO. 1) isoforms were synthesized using a 433A peptide synthesizer (PE Applied Biosystems, Inc., Norwalk, CT).

ASP:1-DTLDEAERQWKAEFHRWSSYMVHWKNQFDHYSKQDRCSDL-40

ARP: 1-GMQGPAGSGWEEGSGSPPGVTPLFSP-26

Length and integrity of the peptide preparations were ensured following purification by HPLC, using a D-6000 chromatography data station (Hitachi Instruments, Inc., San Jose, CA). The working concentrations were 50 and 100 ng/mL (supplemented every 4 days) for liquid cultures grown between 24 hr to 28 days.

Culture analyses: Twenty-four hr liquid cultures served for cytochemical staining, *in situ* hybridization, and cell proliferation assay by 5-bromo-2'-deoxy-Uridine (BrdU) incorporation (as in Grisaru *et al.*, Mol. Cell. Biol. 19, 788-95, 1999). Twenty-eight day liquid cultures were sampled every 6-8 days for viable cell counting (using trypan blue dye exclusion), cell phenotyping ($CD34^+$, $CD33^+$ and $CD41^+$ quantification using flow cytometry, with CD34-PE, CD33-FITC (Immunotech/Coulter, Hialeah, FL), CD41-FITC (Immunoquality Products, Groningen, Netherlands) and CD45-FITC monoclonal antibodies), and growth of progenitor colony (granulocyte-macrophage and megakaryocytic), using previously described techniques (Pick *et al.*, Br. J. Haematol. 103, 639-50, 1998).

Cytochemical staining: Staining of AChE activity was essentially as detailed elsewhere (Grisaru *et al.*, Mol. Cell. Biol. 19, 788-95, 1999), on non-fixed liquid cultures following 300xg centrifugation on collagen-coated cover slips placed on the bottom of the culture well, in the presence of 10^{-5} M iso-OMPA (ISO) or BW284C51 (BW), selective inhibitors of BuChE and AChE, respectively (see also Keymer *et al.*, Eur. J. Neurosci. 11, 1049-57, 1999). Nuclear staining was with 4',6-diamidino-2-phenylindole (DAPI, see e.g., Peterson *et al.*, Genetics 152, 427-439, 1999).

***In situ* hybridization:** *In situ* hybridization procedures, were performed on cultured cells and human fetal tissues, as detailed elsewhere (Grisaru *et al.*, Mol. Cell. Biol. 19, 788-95, 1999, Kaufer *et al.*, Nature 393, 373-7, 1998). Cultured cells were centrifuged at 300 X g and fixed, using

4% paraformaldehyde, to collagen-coated cover slips placed on the bottom of the culture well. Tissues from fetal hematopoietic organs (AGM, liver, spleen and bone marrow) were obtained in each of the selected gestational stages, from 2-3 normal aborted human fetuses. The project was approved by the Sourasky Medical Center Ethics Committee, and written informed consent was obtained from the parents. 5'-Biotinylated, 2'-O-methylated AChEcRNA probes complementary to 3'-alternative human *ACHE* exons were employed. Detection and quantification of the various AChEmRNA transcripts in fetal tissues were performed as previously described (Grisaru *et al.*, Mol. Cell. Biol. 19, 788-95, 1999). Confocal microscopy scans of the culture-derived cells were obtained using a MRC-1024 Bio-Rad confocal microscope (Hemel Hempsted Herts., UK). A projection was built from each cell image and specific criteria were set for size and intensity of the Fast Red fluorescence. Image-Pro 3.0 software (Media Cybernetics, Silver Spring, MD, USA) was used to analyze the signals obtained. ANOVA (Analysis of Variance) test was used for calculation of p values.

DNA sequence analysis: The reverse sequence of the 7q22 cosmid insert (accession no. AF002993) containing the human *ACHE* gene and its upstream sequences, was searched for consensus motifs for binding transcription factors which regulate hematopoietic expression, using the MatInspector program with core similarity of 1, or the Findpatterns program of the University of Wisconsin GCG software package (Quandt *et al.*, Nucleic Acids Res. 23, 4878-84, 1995).

Immunoblot: Mouse serum was diluted 1:10. ARP, ASP, recombinant AChE-S (Sigma Chemical Co.) and recombinant AChE-R extracted from transfected COS cells served as positive controls. Protein electrophoresis in SDS gradient (4-20%) polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) was followed by immunodetection using the rabbit anti-GST-ARP antibodies, Peroxidase-conjugated anti-rabbit immunoglobulins and ECL™ detection (Amersham Pharmacia Biotech, UK).

Methods for culturing murine hematopoietic cells:

Cell Collection:

Blood: 1 ml of murine blood is collected by cardiac puncture and immediately deposited into pediatric vacutainer tubes containing Na citrate. Blood counts are performed using the AcT diff Coulter counter (Coulter-Beckman).

Bone marrow: The tibia and femurs are surgically removed, cleaned and both ends of the bone cut open. The bones are placed in small tissue culture plates containing 2-5 ml of medium composed of RPMI, antibiotics and 10% heat inactivated fetal calf serum (complete medium) supplemented with heparin (5 U/ml) to prevent clotting. The BM contents are flushed out into the medium using a 25 gauge needle. Cells are passed up and down through the syringe three times to guarantee a single cell suspension.

Spleen: The spleen is surgically removed and cleaned and placed in 5 ml of medium composed of complete RPMI with heparin (5 U/ml) as

described above. Both ends of the spleen are cut open and the spleen cells are expressed from the organ capsule by squeezing down on the spleen with the back barrel of a 5 cc sterile syringe. A single cell suspension is prepared by passing the cells up and down three times through a 5 ml syringe.

Cryopreservation:

The hematopoietic cells are washed by the addition of an additional 5 ml of medium composed of complete RPMI with heparin, counted by manual hemocytometry and pelleted at 1500 RPM for 10 minutes. The cell pellets are resuspended at a concentration of 5X 10^6-10^7 cells/ml in ice cold freezing medium containing 50% DMSO and 30% RPMI (as above). Cells are allowed to remain on ice for up to 5 minutes and subsequently placed at -20°C for 30 minutes. Cells are then transferred to -80°C for a period of up to 6 months; for longer storage, cells are transferred to -180°C.

Hematopoietic progenitor cultures

For all colony assays duplicate samples of 1-2x10^5 cells are placed into 1 ml of medium plus the appropriate supplements for each cell lineage in small round tissue culture dishes (2.5 cm). These small dishes are placed into a larger TC dish (10 cm) together with a third small dish containing sterile water to prevent evaporation in the cultures. Cells are cultured at 37°C in a fully humidified atmosphere containing 5% CO_2.

BFU-E

Two hundred thousand ($2x10^5$) cells are placed into 1 ml of Alpha medium containing antibiotics, 30% methyl cellulose, 10% FCS and 2ng/ml recombinant murine (r-mu) erythropoietin, and both BFU-E and the smaller CFU-E are counted after 10 days.

CFU-GM and CFU-GEMM

One hundred thousand ($1x10^5$) cells are placed into 1 ml of Alpha medium containing antibiotics, 3% agar, 10% FCS and 5 ng/ml or both r-mu IL-3 and r-mu GM-CSF. CFU-GM, CFU-GEMM and CFU-bl are counted after 12 days.

CFU-MK

Two hundred thousand ($2x10^5$) cells are placed into 1 ml of Alpha medium containing antibiotics, 30% methyl cellulose, 10% FCS and 5ng/ml. r-mu thrombopoietin and 10 ng/ml stem cell factor. CFU-MK and BFU-MK are counted by staining the cells for AChE after 12 days.

Animal models and in vivo experiments - transgenic FVB/N mouse pedigrees expressing human AChE variants were described elsewhere, as were the biochemical methods for measuring AChE activity (Sternfeld *et al.*, J. Physiol., Paris, 92, 249-55, 1998). The confined swim protocol for exerting acute psychological stress was performed as detailed (Kaufer *et al.*, Nature 393, 373-7, 1998). Immediately following the stress, the treated

mice were injected intraperitoneally with 100 ng ARP or 0.03 ng AS1, both per gram body weight. Another group of non-stressed mice were injected either with normal saline or ARP. Twenty-four hours later, the animals were sacrificed and peripheral blood was collected in EDTA covered tubes (Becton Dickinson Immunocytochemistry System, Inc., San Jose, CA) prepared with 25 units of heparin sodium USP (Kamada LTD, Kibbutz Beit-Kama, Israel). Whole blood AChE activity was analyzed, and WBC and platelet counts determined, using an Ac·T diff hematology analyzer (Beckman Coulter, Inc., Fullerton, CA).

Cytochemical and immunohistochemical staining - staining of AChE activity was as detailed above. For immunohistochemistry, murine bone marrow smears were fixed with 4% paraformaldehyde (10 minutes, room temperature, RT); permeabilized with buffer containing 20 mM HEPES (pH 7.4), 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$ and 0.5% Triton X-100 (4 minutes on ice); washed twice with PBS (5 min each, RT); incubated in 1% H_2O_2 in methanol (15 min RT); and washed twice in PBS. Non-specific sites were blocked by incubating in 5% horse serum in PBS (20 min, RT). Labeling was in a humidified chamber with 1:50 dilution of affinity purified rabbit antiserum prepared against GST-fused recombinant ARP (1 hr, RT). Following 3 washes with PBS, smears were incubated with 1:100 biotinylated goat anti-rabbit Ig (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) (30 min, RT). After 3 PBS washes, a mixture of biotin and avidin-peroxidase was added (30 min,

ABC Elite Kit, Vector Labs, Burlingame, CA) and reacted with diaminobenzidine-hydrogen peroxide mixture (Sigma Chemical Co., St. Louis, MO, 10 min), followed by counterstaining with Meyer's hematoxylin mixture (Sigma Chemical Co., St. Louis, MO), and immunomounting.

Expression of recombinant ARP - The sequence, coding the C-terminal region of I4 (i.e., the "readthrough" variant of acetylcholinesterase, comprising the ARP peptide sequence), was amplified by PCR using the following oligonucleotide primers:
GCT GGA TCC ATC GAG GGG CGA GGT ATG CAG GGG CCA GCG GGC (I4-up), also denoted as SEQ ID No. 4,
and TAT AAG CTT CTA GGG GGA GAA GAG AGG GGT (I4-down), also denoted as SEQ ID No. 5, and introduced into pGEX-KG (ATCC accession No. ATCC77103, see also Anal. Biochem. 192:262-267, 1991) plasmid.

Antibody production

GST and I4-GST fusion protein were purified from the supernatant of E. coli lysate by affinity chromatography on glutathione-Sepharose (Pharmacia), eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, dialyzed to 0.1 M ammonium acetate buffer, pH 7.0, aliquoted and lyophilized. The stability and identity of the protein was confirmed by SDS-PAGE. The following protease inhibitors were used during the preparation: aprotinin (10 microgram/ml), benzamidine (5 mM), Pefabloc SC (0.2 mM), and EDTA (1 mM). Prior to affinity chromatography, the E.

coli lysate was incubated for 20 min at 37°C with 0.2 mM Mg-ATP in order to dissociate the fusion proteins from contamination of bacterial proteins. The procedure was performed according to Pharmacia recommendations.

Two New Zealand female rabbits were immunized subcutaneously with 0.3 mg fusion protein in complete Freund's adjuvant, and then reimmunized monthly with 0.2 mg fusion protein in incomplete Freund's adjuvant. Blood samples were taken 10 days after the immunization. The specific antibodies in the sera were detected by ELISA on immobilized fusion protein, in the presence of excess of soluble GST (20 microgram/ml). The reacting sera were chosen for antibody purification. The immobilized I4-GST, GST and E. coli lysate were prepared using Affigel 10 (Bio-Rad) according to the manufacturer's recommendations.

Crude IgG fraction was prepared from the serum by 50% saturation $(NH_4)_2SO_4$ precipitation and dialyzed in 100 mM Tris-HCl, pH 8.0. In order to get rid of anti-GST antibodies, the IgG fraction was incubated with GST beads (Affigel 10, Bio-Rad) overnight at 4°C. The bound material was eluted with 4.5 M $MgCl_2$. The procedure was repeated with the unbound material several times, until no antibodies were eluted from GST beads. In order to get rid of antibodies against possible contamination of bacterial proteins, the same procedure was performed with immobilized heat-shocked E. coli lysate proteins.

The unbound material was then applied to I4-GST beads (Affigel 10, Bio-Rad), incubated 2 hr at room temperature or overnight at 4°C, and the bound material was eluted with 3.5 M $MgCl_2$. The eluted antibodies were

dialyzed against 10 mM Tris-HCl, pH 8.0, and then against PBS, containing 0.025% NaN_3.

Example 1

Hydrocortisone elevates *ACHE* gene expression in hematopoietic stem cells

This example relates to *ACHE* gene expression in hematopoietic stem cells and the influence of hydrocortisone thereon. The inventors have searched the extended promoter of the human *ACHE* gene (cosmid accession no. AF002993) for consensus motifs that may bind stress-associated and hematopoietic transcription factors. Two such clusters, one located 17 Kb upstream from the transcription start site, and another positioned at the first intron, were found to include motifs for AP1, NFkB, EGR-1 (as identified by a matrix search against the TransFac database, Heinemeyer *et al.*, Nucleic Acids Res. 26, 364-370, 1998), interleukin-6 (IL6), with the consensus sequence CTGGG/AAA, glucocorticoid responsive element (GRE) half palindromic site, TGTTCT, and Stat-5, TTCCCAGAA or TT(C/A)(C/T)N(A/G) (G/T)AA (Figure 1A). Of these, the latter two motifs are known to be actively involved in hematopoiesis (Darnell *et al.*, Science 264, 1415-21, 1994) and cellular stress responses (Tronche *et al.*, Curr. Opin. Genet. Dev. 8, 532-8, 1998). Moreover, they act synergistically in enhancement of β-casein gene expression in hematopoietic cells (Lechner *et al.*, Immunobiology 198,

112-23, 1997) but have not yet been studied in the context of AChE involvement in hematopoiesis.

Fig. 1 A is a scheme of the upstream human *ACHE* sequence including clusters of hematopoietic and stress-related motifs. Depicted is a scheme of the reverse sequence of the cosmid insert (accession No. AF002993) of the human *ACHE* promoter. The arrow represents the position of a transcription start site. Two potentially relevant regions are shown, one beginning at nucleotide 5267 and one following the first exon (black box). Fully conserved consensus sequences are marked by triangles. These include AP-1, NF-κB, EGR-1, IL-6, glucocorticoid responsive element (GRE) half-palindromic site, and Stat-5.

The functional effects of the glucocorticoid-binding motifs in the *ACHE* upstream sequence (Figure 1A) were investigated in human UCB $CD34^+$ stem cells isolated by anti-CD34-coated immunobeads to yield a 85 ± 3% pure population, as confirmed by flow cytometry. $CD34^+$ cells were enriched from human UCB cells using bead-attached antibodies to the CD34 protein. Fig. 1 B shows a representative flow cytometry of the recovered cells, demonstrating that 89% of them express the CD34 antigen. The inset in Fig. 1B shows an example photograph of enriched $CD34^+$ cells stained by May-Grünwald-Giemsa. Note the large nuclei surrounded by thin rims of cytoplasm, characteristic of stem cells.

Enriched $CD34^+$ cells were subjected to cytochemical staining for AChE catalytic activity in the presence of 10^{-5} M iso-OMPA (Fig. 1C, ISO) or BW284C51 (Fig. 1C, BW), selective inhibitors of BuChE and AChE,

respectively. Nuclear staining (Fig. 1C, right) was with DAPI. Note the selective appearance of brown precipitates of AChE, but not BuChE reaction products. The data shown in Fig. 1C demonstrate that $CD34^+$ cells contain cytochemically detectable levels of catalytically active AChE. The identity of their cholinesterase as AChE was verified by its sensitivity to the AChE-specific inhibitor BW284C51 and its resistance to the butyrylcholinesterase (BuChE) inhibitor iso-OMPA (Fig. 1C).

The assumption that hematopoietic *ACHE* gene expression is modulated under stress was tested in $CD34^+$ cells cultured for 24 hr with increasing doses of hydrocortisone. Treated cells were subjected to cytochemical staining for AChE activity as well as to high resolution *in situ* hybridization followed by confocal microscopic quantification of labeling density. This method provides an accurate and credible tool for the examination of transcriptional responses in the heterogeneous population of primary HSCs from different individuals. Hybridizations were performed for each of the three transcripts of human AChEmRNA presented in Figure 1A (S, E and R). Because each cRNA probe has its own characteristic hybridization affinity, each transcript was quantified separately. Individual $CD34^+$ cells were treated with the noted doses of hydrocortisone at levels equivalent to physiologically normal, intermediate and stress conditions (0.1, 0.6 and 1.2 μM, respectively; see De Vroede *et al.*, Arch. Dis. Child 78, 544-7, 1998). Cells were subjected to *in situ* hybridization with the noted AChEcRNA probes, followed by confocal

microscopy, projection of image slices, quantification and color-coding of the labeling signals.

Fig. 1D presents cytochemically stained cells (top) and representations of 3-dimensional projections created from confocally scanned sections of $CD34^+$ cells following *in situ* hybridization with 5'-biotinylated AChEcRNA probes selective for the "synaptic" AChE-S mRNA variant, the "erythrocytic" AChE-E mRNA variant encoding for glycophospholipid-anchored AChE-E and the "readthrough" AChE-R mRNA form associated with stress. Detection was by appearance of Fast Red precipitates (Grisaru *et al.*, Mol. Cell. Biol. 19, 788-95, 1999). Note increasing cytoplasmic labeling under high hydrocortisone levels. Each photograph represents one out of 10-20 analyzed cells with deviations in labeling of less than 6%. Fig. 1D, left, bottom shows the relative increases in percent above control for each of the analyzed transcripts under stress-relevant concentrations. Note the accumulation of AChE-R mRNA transcripts under moderate hydrocortisone concentrations.

Fig. 1D demonstrates that subtle elevation of hydrocortisone concentration to 0.60 μM induced a 40% selective increase in the "readthrough" AChE-R mRNA transcript above the level observed under non-stress hydrocortisone concentration (0.10 μM) (De Vroede *et al.*, Arch. Dis. Child 78, 544-7, 1998). However, at 0.60 μM hydrocortisone, no change was observed in enzyme activity of $CD34^+$ cells. In contrast, stress-associated hydrocortisone levels (1.2 μM) enhanced the labeling of all 3 AChEmRNA transcripts and intensified the catalytic activity of the

stem cell-associated enzyme. The AChE-R mRNA-specific *in situ* hybridization, therefore detects a clear increase in this variant against a low background, while total cell-bound enzyme activity registers no deviation from background at a sub-stress hydrocortisone level.

Example 2

Readthrough AChE is overproduced in the myeloidogenic mid-gestation liver

To study the relevance of each of the AChEmRNA transcripts during development of the hematopoietic organs, *in situ* hybridization was performed on paraffin-embedded sections taken from human fetuses at different gestational ages. Consistent with the embryonic spatiotemporal shifts in blood cell forming tissues, we observed changes in the labeling intensity with the various probes used, in the aorta-gonad-mesonephric region (AGM), liver, spleen and bone marrow cells. Figure 2A schematically presents the migration of hematopoiesis between the various blood cell forming tissues during fetal development. The top left of the figure represents a sagittal section of a human embryo showing the hematopoietic organs – AGM (aorta-gonad-mesonephros), LIV (liver), SPL (spleen), and BM (bone marrow). The top right of the figure is a scheme of gestational shifts in hematopoietic processes which shows the relative intensity of blood cell formation in the various hematopoietic organs throughout human gestation. (according to Tavassoli *et al.*, Blood Cells 17, 269-81, 1991, Tavian *et al.* Development 126, 793-803, 1999). Ages of

embryos on which *in situ* hybridization was performed are marked by gray columns.

Fig. 2B presents *in situ* hybridization results and the average labeling intensities for the AChE-S, AChE-E and AChE-R mRNA transcripts in AGM (triangles, week 9), liver (diamonds), spleen (squares) and bone marrow (triangles, weeks 20-25) of human fetuses at different gestational ages (right side curves). The figure shows representative *in situ* hybridization micrographs from the noted tissues of human fetuses at the noted gestational ages, using selective probes for each of the above alternative human AChEmRNA transcripts. The right side of the figure shows spatiotemporal changes in labeling intensity for each probe and organ. Note that AChEmRNA expression increases parallel to active hematopoiesis in the examined organs.

For the AChE-S and AChE-E probes, expression levels were distributed similarly in liver and spleen. For example, labeling intensity for both these probes was high in mid-gestation liver and spleen, when the principal hematopoietic activity was erythropoiesis, and labeling of both decreased steadily from the 9th week onward, as myelopoiesis became more prevalent. In contrast, the AChE-R transcript was detected only during the 16 week transition from erythro- to myelopoiesis in the mid-gestation liver and not in the spleen (Porcellini *et al.*, Int. J. Cell Cloning 1, 92-104, 1983). The unique expression pattern of AChE-R mRNA and its apparent correlation with myeloidogenesis demonstrated that AChE-R acts as a selective hematopoietic element.

Example 3

ARP sustains cell expansion and differentiation

The predicted secondary structure of peptides ARP and ASP was analyzed. Fig. 3 A presents the amino acid sequences of ARP and ASP (26 and 40 residues, respectively). Secondary structure predicted using the peptide structure program of the GCG software package (University of Wisconsin) was based on the Chou-Fasman method. Depicted below the sequences are the secondary structures predicted: T, turn, B, β-sheet and H, α-helix, with lower case letters representing lower predicted probability. Note the predicted helix structure for the first 17 residues of ASP, drawn using the Helicalwheel program of the GCG software package. The amphipathic nature of this region is postulated based on the unilateral positioning of hydrophobic residues (F, L, W, W, A).

Both the "synaptic" exon 6-derived and the "readthrough" pseudointron 4-derived peptides (ASP, ARP) include a major region predicted to be rich in turns and β-pleated sheets; in addition, the longer ASP peptide is predicted to contain a unilaterally hydrophobic α-helical domain with amphipathic properties (Figure 3A). To test whether either of these peptides has biological activity, the inventors added HPLC-purified synthetic peptides (in 50 and 100 ng/ml final concentrations) once every 4 days to the growth medium in which isolated HSCs ($CD34^+$) were cultured for 2 weeks. Fig. 3B shows fold expansion values of viable cells, based on trypan blue exclusion (average of 4-5 experiments ± standard error of the

mean, SEM) grown for 2 weeks in the presence of the noted growth factor or mixtures of factors and, where marked, 50 ng/ml ASP or ARP. Asterisks note statistical significance of the measured increases in cell counts as compared to cultures without the peptide ($p \leq 0.05$).

Addition of ARP alone increased the number of viable cells more than 10-fold (Figure 3B; $p<0.008$). ARP further improved expansion of viable cells when it was administered in combination with SCF, granulocyte-macrophage colony stimulating factor (GM-CSF), or thrombopoietin (TPO). However, its growth factor-accessory effect reached statistical significance only with TPO ($p<0.01$). Similar doses of ASP were less effective than ARP in promoting cell expansion, alone or when added with any combination of these cytokines for 2 weeks (Figure 3B). The compatibility of the ARP-supported expansion with differentiation was tested by quantifying the myeloid ($CD33^+$) and megakaryocytic (MK, $CD41^+$) cells after 2 weeks in liquid culture (Table 1). Cells expanded under the influence of ARP displayed increased ability to differentiate into MK and myeloid progeny. Moreover, ARP potentiated the effect of TPO to enhance the number of MKs. Surprisingly, the TPO-potentiating effect of ARP was found to be more pronounced than the TPO-potentiating effect of stem cell factor (SCF). SCF is a known TPO potentiating agent for stem cells (Deutsch *et al.*, Med. Oncol. 13, 31-42, 1996). ARP also facilitated the capacity of GM-CSF and SCF to support myelopoiesis. Thus, the effects of ARP over MK ($p=0.08$, paired Student's t-Test) and myeloid ($p=0.04$) expansion are independent. Further, the effect of ARP on the

megakaryocytopoietic capacity of TPO and SCF (p=0.03) is synergistic. The myeloid potentiation capacity of ARP over that of GM-CSF and SCF is additive.

Table 1

ARP potentiates TPO, GM-CSF and SCF effects on megakaryocytic and myeloid lineages[a]

cell type / cytokine	MK ($CD41^+$)	Myeloid ($CD33^+$)
None	0.03 ± 0.02	1.50 ± 0.79
ARP	**8.20 ± 4.50**	**2.90 ± 0.65**
TPO	3.93 ± 3.56	3.51 ± 2.23
TPO + ARP	**48.43 ± 34.94**	2.95 ± 0.64
GM-CSF	14.50	1.35
GM-CSF + ARP	7.80	**1.98**
SCF	0.30 ± 0.21	3.90
SCF + ARP	**0.90 ± 0.27**	6.49
SCF + TPO	20.60 ± 20.04	0.68

[a]Presented are fold expansions (and, were noted, SEMs) of 2-week primary cultures of $CD34^+$ cells from 1 to 3 individuals grown with the noted cytokines. ARP, AChE C-terminal "readthrough" peptide; TPO, thrombopoietin; GM-CSF, granulocyte-macrophage colony stimulating factor; SCF, stem cell factor; MK, megakaryocyte. Potentiated expansion values are highlighted in bold letters.

Example 4

Autoregulatory effect of ARP

ARP was added in concentrations of 50 or 100 ng/ml to cultured $CD34^+$ cells and the levels of the various AChEmRNA transcripts after 24 hr were examined, by *in situ* hybridization combined with confocal microscopy analysis. Fig. 4 (left) shows representative individual $CD34^+$ cells treated for 24 hr with the noted doses of ARP in the absence of other growth factors and subjected to *in situ* hybridization with probes selective for each of the alternatively spliced variants of AChEmRNA. The right side of the figure shows average labeling densities for 10-20 cells in each case. Fig. 4 demonstrates similar increases for all 3 transcripts (S, E ,R) with peak activity at 50 ng/ml ARP. Note the concomitant increases in all transcripts, and the uniform nature of this response in all of the analyzed cells. This suggests that ARP stimulates transcriptional enhancement of the *ACHE* gene. Autoregulatory continuation of AChE-R production could sustain the ARP effect long after the initial ARP signal has been terminated.

Example 5

ARP retrieves the antisense-suppressed cell proliferation effect of GM-CSF

Antisense suppression of AChE-R production - The ARP-induced enhancement of *ACHE* gene expression suggested that the AChE-R protein and not necessarily ARP, may be responsible for the sustained viability and the significant expansion of HSCs. To distinguish between these two

possibilities, the inventors employed antisense oligodeoxynucleotides (AS-ODN) to selectively suppress AChE-R mRNA levels, reduce the intracellular production of AChE-R and test, under these conditions, the proliferative effects of GM-CSF with or without ARP. AChE-R mRNA includes a 1,094 bp 3'-untranslated region (UTR), with 62% G,C content. This marks it as a more vulnerable molecule to nucleolytic degradation than AChE-S mRNA, which includes a 219 bp UTR with 66% G,C. A. Fig. 5A shows AS-ODNs targeted to the common sequence domain of mRNA transcripts with variable UTRs. Shown are schematic structures of the two human cholinesterase genes, *ACHE* and *BCHE*. Exons are colored or gray, introns are shown in white. The open reading frame (ORF) is drawn above each gene, and the positions and predicted structures of the AS-ODNs that were employed are drawn below. Also marked are the UTRs for the two AChEmRNA transcripts, AChE-S (UTR = 219 bp, 66% G, C) and AChE-R (UTR = 1,094 bp, 62% G, C).

To selectively reduce AChE-R mRNA levels in HSCs, extremely low doses (20 pM) of anti-AChEmRNA AS-ODNs (Grisaru *et al.*, Mol. Cell. Biol. 19, 788-95, 1999) were employed. AS1 and AS3 are 2'-O-methyl-protected AS-ODNs targeted to *ACHE* exon 2, which is common for AChE-S and AChE-R mRNA. An irrelevant AS-ODN (ASB) targeted to BuChE mRNA served as a control (Figure 5A and Grisaru *et al.*, Mol. Cell. Biol. 19, 788-95, 1999). Fig. 5 B shows selective susceptibility of AChE-R mRNA to AS-ODN destruction. $CD34^+$ stem cells were treated for 24 hr at 37°C with 20 pM 2'-O-methylated AS-ODNs targeted to AChEmRNA or

BuChEmRNA. Shown are DAPI and AChE activity stainings (left) and confocal images of *in situ* hybridizations for the AChE-S and AChE-R transcripts (right) with 20 pM of the ASB, AS1 or AS3 AS-ODNs. Columns show average levels of staining efficiencies for 10-20 cells hybridized with each of the transcript-specific probes. Note maintenance of cell-associated AChE activities and stable levels of AChE-S mRNA under all treatments as opposed to selective reduction of AChE-R mRNA under AS1 treatment. Thus, AS1, but not AS3 reduced the *in situ* detected AChE-R mRNA levels in $CD34^+$ cells under conditions where AChE-S mRNA levels remained unchanged (Figure 5B). The irrelevant ASB ODN was ineffective, demonstrating sequence specificity of the AS1 effect.

ARP retrieves the antisense-suppressed cell proliferation effect of GM-CSF - Cell proliferation was evaluated by measuring BrdU incorporation following 16 hr incubation in the presence of 20 pM of the noted AS-ODNs with or without 50 ng/ml ARP and/or GM-CSF. Fig. 5 C shows average results of 3-6 reproducible experiments ± SEM. Consistent with its expansion effect, incubation with GM-CSF increased the incorporation of bromodeoxyuracil (BrdU) into $CD34^+$ cells over 16 hr (Fig. 5C). Addition of 50 ng/ml ARP together with GM-CSF significantly potentiated this incorporation ($p<0.03$), whereas ARP, AS1, AS3 or ASB did not affect BrdU incorporation when added alone to the cells (Fig. 5C). The capacity of GM-CSF to enhance BrdU incorporation was totally suppressed when it was added together with 20 pM AS1. The suppressive effect of AS3 on GM-CSF-induced enhancement of BrdU incorporation, was

much weaker than that of AS1, consistent with its inability to suppress AChE-R mRNA levels in $CD34^+$ cells. To examine whether ARP alone was required and sufficient to facilitate the cell proliferation effect of GM-CSF, the inventors incubated the cells with GM-CSF and ARP together with the suppressive AS1. ARP completely reversed the AS1-induced suppression in BrdU incorporation, retrieving the full capacity of GM-CSF to enhance cell proliferation (Fig. 5C). Thus, the data show that ARP enhances the GM-CSF-supported increases in cell proliferation, that AS1 reduces this enhancement far more effectively than AS3, and that ARP retrieves the AS1-suppressed proliferation.

Example 6

ARP can substitute for stem cell factor

To determine whether the ARP expansion effects could replace any of the known growth factors, the inventors tested ARP alone or combined with known growth factors, on long-term $CD34^+$ cell cultures. Fig. 6A shows cell counts from long-term $CD34^+$ liquid cultures grown in the absence of growth factors (blue, diamonds), in the presence of early-acting cytokines (EAC: IL3, IL6, TPO and FLT3) and SCF (green, squares), or in the presence of EAC + ARP with SCF (black, circles) or in the presence of EAC + ARP without SCF (red, triangles). Viable cell counts are depicted in the upper left part of Fig. 6A. CD34+ cell counts are presented in the upper right part of the figure. The lower left and right parts are graphs of the

number of Granulocyte-Marcophage (GM) or Megakaryocyte (MK) progenitor colony forming units.

Fig. 6A, upper left, shows that early-acting cytokines (a mixture of IL3, IL6, TPO and FLT3) promote linear expansion of $CD34^+$ cells for up to 28 days. In the absence of this mixture, there was no proliferation. SCF, although devoid of proliferative activity by itself, enhances significantly the proliferation induced by the above growth factors (Li and Johnson, Blood 84, 408-14, 1994). Addition of ARP, with or without SCF resulted in an enhanced cellular proliferation, leading in both cases to a greater than 2000-fold expansion within 28 days (Fig. 6A, upper left). This demonstrates that the activity of ARP was additive to that of the early-acting cytokines and that it could replace SCF.

Fig. 6A, upper right, shows that ARP operates as a $CD34^+$ survival factor. Note that $CD34^+$ cell numbers reach a plateau at 21 days in the presence of EAC (squares), and that ARP facilitates further increases in $CD34^+$ counts up to at least 28 days, regardless of the presence of SCF (triangles, circles). Thus, the conclusions drawn above from the results of Fig. 6 A, upper left, are supported by the finding that ARP with or without SCF promoted, with similar efficacy, the survival of $CD34^+$ cells within the expanded cultures as compared with survival in the absence of growth factors (Fig. 6A, upper right).

Fig. 6A, lower part, show that ARP increases the number of GM and MK progenitors. Shown are counts of colony forming units for GM (left) or MK (right) colonies grown from progenitors removed at 13, 21 and 28 days

of the primary expansion phase detailed under Figure 6A. The numbers of colonies grown after EAC, EAC + SCF + ARP or EAC + ARP treatment are very similar, suggesting redundant expansion properties for SCF and ARP.

Fig. 6B shows that ARP facilitates development of hematon bodies. Representative photographs of the 28-day liquid cultures detailed in Figure 6A above are shown. In the absence of growth factors, sparse hematopoietic cells and many fibroblasts are seen (control, upper left). Addition of EAC increases the density of small, round hematopoietic stem cells and sparse MKs (Fig. 6B, upper right, white arrow). Fig. 6B, lower half, demonstrates that EAC + ARP facilitate the formation of hematon bodies (insets) without (right) or with SCF (left).

Fig. 6A, lower part, shows that ARP increases the number of GM and MK progenitors. Shown are counts of colony forming units for GM (left) or MK (right) colonies grown from progenitors removed at 13, 21 and 28 days of the primary expansion phase detailed under Figure 6A. The numbers of colonies grown after EAC, EAC + SCF + ARP or EAC + ARP treatment are very similar, suggesting redundant expansion properties for SCF and ARP.

In summary, $CD34^+$ cultures grown without growth factors for 28 days displayed typical fibroblast morphology (Fig. 6B, Upper left). In contrast, a dense population of small, round cells, with characteristic stem cell morphology, was observed in cultures grown for the same period in the presence of the early-acting cytokines (Figure 6B, Upper right). The addition of ARP, in the presence or absence of SCF, sustained this stem cell morphology (Figures 6B, Lower left and right). Interestingly, floating

"hematons", which are independent hematopoietic units rich in myeloid, erythroid and megakaryocyte progenitor cells (Blazsek *et al.*, Exp. Hematol. 23, 309-19, 1995) were found in the ARP-containing cultures, demonstrating the differentiation potential of this peptide (Fig. 6B, lower part, insets).

Example 7

ARP-treated cells maintain multipotent progenitor properties

To test the number of progenitors and differentiation routes available to ARP-treated cells, the inventors subjected the above cultures to a second expansion phase. Cells removed once a week from the primary liquid cultures were grown in the absence of ARP in a semi-solid substrate. In the absence of the growth factor mixture, there was no secondary expansion. IL3 and GM-CSF were used to induce granulocyte-macrophage (GM) expansion and TPO and SCF was used for megakaryocyte (MK) expansion. During this second expansion phase, blood cell progenitors that had previously been treated with early-acting cytokines developed into either GM or MK colonies (Fig. 6B, lower part), depending upon the added growth factor. The numbers of GM and MK colonies peaked by 3 weeks and were essentially the same in cultures that were previously treated with all of the early acting cytokines, with or without ARP. ARP-supported hematopoiesis thus appeared to maintain normal growth of differentiated myeloid and megakaryocyte colonies.

Example 8

in-vivo ARP effects

ARP accumulates in the serum under stress and facilitates the stress-induced hematopoietic responses in vivo - To find out whether the ARP peptide occurs naturally in blood and if its levels increase under psychological stress, FVB/N mice (n=12) were subjected to confined swim protocol for exerting acute psychological stress as detailed elsewhere (Kaufer *et al.*, Nature 393, 373-7, 1998). Serum samples removed 24 hr later were subjected to gradient gel electrophoresis. Fig. 7A, top, shows a Poinceau-stained polyacrylamide gradient gel (4-20%, Bio-Rad) loaded with: (1) protein extract from COS cells transfected with AChE-R encoding plasmid (Ben Aziz-Aloya et al., Proc. Natl. Acad. Sci. USA 90, 2471-5, 1993, Seidman et al., Mol. Cell. Biol. 15, 2993-3002, 1995) and mixed with synthetic ARP (ARP+AChE-R); (2) recombinant AChE-S (Sigma), mixed with synthetic ASP (ASP+AChE-S); (3) serum (2 μL) from a saline-injected mouse, removed 24 hr post-treatment (Control); (4) serum from a mouse subjected to confined-swim stress as described above, removed 24 hr post-treatment (Stress). Positions of molecular weight markers are shown on the left. The gel was then electroblotted and immunodetected (see "immunoblot" in the Experimental Procedures section for details) with affinity-purified rabbit antibodies elicited toward a recombinant GST-ARP fusion protein (Fig. 7A, bottom). A 67 KDa protein, consistent with the expected size of AChE-R, is detected in the serum (upper arrow).

Furthermore, selective labeling of synthetic ARP (but not AChE-S or ASP) by this antibody is detected. Accumulation of ARP in the serum of stressed mice is evident from the intense labeling of native ARP in the stressed mouse serum (lower arrow).

To determine the *in vivo* capacity of ARP to affect hematopoietic expansion under acute psychological trauma, mice were injected immediately after the stress protocol with 0.1 mg/kg ARP or 30 ng/kg AS1. Another group of mice were not subjected to stress and were injected intraperitoneally with normal saline (n=6) or ARP (n=4). 24 hours later, the animals were sacrificed and whole blood obtained for AChE activity and white blood cells. Bone marrow smears were subjected to immunohistochemical labeling with an affinity purified rabbit antiserum prepared against GST-fused recombinant ARP. Fig. 7B shows the number of labeled cells per 100 cells counted at x1000 magnification in 5 different fields. Bone-marrow labeling and white blood cell (WBC) count were similar in non-stressed mice regardless of ARP injection. In contrast, ARP intensified labeling and increased the number of small positive cells in the bone marrow of stressed mice, indicating that it enhances AChE expression and increases stem cell expansion *in vivo*. AS1 reduced the number of cells labeled with anti-ARP antibodies (Fig. 7B). In peripheral blood, WBC counts revealed similar ARP-dependent enhancement and AS1 suppression.

Persistent AChE-R overproduction increases platelet and WBC counts in a dose-dependent manner - A series of AChE transgenic mouse

pedigrees (Sternfeld *et al.*, J. Physiol., Paris, 92, 249-55, 1998) was employed, to reveal if chronic increases in AChE-R would confer persistent changes in blood cell composition. Blood AChE levels, platelet and WBC counts were determined in FVB/N mice (Control, n=22) as compared to transgenic FVB/N mice carrying the AChE-S (TG-S, n=12), AChE-R (TG-R70 and TG-R45, n=9 and 6, respectively) or inert-inactivated AChE-S (AChE-Sin, n=3) transgenes. Fig. 7C shows results expressed as average $\pm$ standard error of the mean (SEM). The transgenic lines expressing AChE-S variants indicated no increases in blood AChE and no significant deviations from a normal blood cell composition. In contrast, increases of 2.5 and 130-fold catalytic AChE activities were observed in two pedigrees (TG-R45 and TG-70R), whereas WBC counts were only increased in the more efficiently overproducing line, suggesting a gene dose dependent effect for ARP over the hematopoietic balance also under chronic excess conditions (Figure 7C).

ARP accumulation in the serum under stress. The intense labeling of ARP in the unfractionated mouse serum removed 24 hr following stress treatment revealed more pronounced increases in this peptide than in its native protein AChE-R. This may reflect elevated proteolytic activity under stress. Combined with the absence of cleavage sites for common proteases within the ARP sequence, this further explains the reproducible series of proteolytic degradation products of serum AChE-R which were intensified in the stressed serum samples. The physiological implications of this finding are that AChE catalytic activity measurements are underestimates

of the extent of its overproduction in the blood under stress. Likewise, measuring acetylcholine hydrolysis may underestimate the actual amounts of the AChE protein and its degradation products in the brain or muscle. The reported decreases of AChE activity in Alzheimer's disease may hence mislead researchers and clinicians alike by masking the accumulation of morphologically active AChE-derived peptides with long-term effects.

ARP modulations potentiate the in vivo hematopoietic responses to stress. While ARP alone did not exert immediate effects on mouse blood cell composition, its injection under stress enhanced ARP labeling in bone marrow cells and induced an elevation in WBC counts within 24 hr. This suggests that acute stress modifies the number and/or state of ARP-responsive elements on hematopoietic cells. Anti-ARP antibodies labeled primarily small cells in ARP-treated stressed animals, whereas the limited labeling in untreated stressed animals and in AS1-treated stressed animals only appeared in relatively larger cells. This indicates labeling of the stem cells which expanded during the 24 hr post-stress. The similar patterns of the *in vivo* effects on bone-marrow ARP labeling and WBC counts with the *ex vivo* expansion effects on $CD34^+$ cells implies that stress-induced increases in AChE-R may be causally related to the post-stress elevation in WBC counts (Goldberg *et al.*, Folia Biol. 36, 319-31, 1990).

Transgenic animal models used here provide an opportunity for testing the chronic effects of elevations of different AChE variants. While AChE-S had no apparent effect on either platelet or WBC counts, AChE-R

modulations exerted dose dependent changes: 2.5-fold excess in blood AChE-R activity, similar to the AChE-R elevation noted in the mouse brain under stress (Kaufer *et al.*, Nature 393, 373-7, 1998) sufficed to significantly elevate platelet counts. The more dramatic 130-fold excess in blood AChE-R levels of the robust-producing transgenic pedigree (Sternfeld *et al.*, J. Physiol. Paris 92, 249-55, 1998) elevated both platelet and WBC counts. This finding, and the *in vivo* accumulation of ARP under stress, raise the possibility that the increased risk for brain infarcts following acute stress or exposure to anticholinesterases (Harmsen *et al.*, Stroke 21, 223-9, 1990, Schultz *et al.*, Anesthesiology 79, 114-21, 1993) is associated with the increased platelet counts due to AChE-R overproduction. This calls for a search for AChE-R overproduction in Alzheimer's disease patients, where ARP may increase platelet counts and cause the cerebral infarcts, characteristic of this disease (Inestrosa *et al.*, Neurosci Lett 163, 8-10, 1993, Snowdon *et al.*, Jama 277, 813-7, 1997). Anti-ARP antibodies provide a novel diagnostic tool for testing this option (and for risk assessment) and AS-ODN treatment may offer an attractive protocol for prevention of such adverse responses.

The significance of ARP extends beyond the hematopoietic system. There is evidence for cross-talk between hematopoietic cells at different stages of differentiation and bone-marrow stromal or endothelial cells. Stroma influences cytokine production and is responsible for maintaining steady-state hematopoiesis and its adjustment under stress (Gupta *et al.*, Blood 91, 3724-33, 1998). It has been proposed that primitive $CD34^+$

progenitors provide a soluble positive feedback signal to induce cytokine production by either stromal or endothelial cells (Jazwiec *et al.*, Leukemia 12, 1210-20, 1998). ARP may play such a role, with important implications for *ex vivo* stem cell expansion, cancer treatment and gene therapy. In the mammalian brain, ARP may further affect the stress-associated plasticity of neuron and glia properties, consistent with previous findings of the inventors of morphogenic activities for AChE-R in transfected glia (Karpel *et al.*, J. Neurochem. 66, 114-23, 1996).

The stem cell survival and proliferative effects of ARP denote a previously unforeseen activity that is particular to the AChE-R protein yet distinct from the Acetylcholine hydrolysis and cell-cell adhesion capacity characteristic of the core domain common to all AChE isoforms. The pronounced expression of AChE-R during early embryogenesis, further demonstrate the involvement of ARP in inducing the proliferation of other embryonic stem cells. Moreover, neural stem cells were shown to produce a variety of blood cell types *in vivo* (Bjornson *et al.* Science 283, 534-7, 1999).

The findings presented here suggest that ARP is involved in the induction of growth and expansion capacities of pluripotent stem cells from multi-tissue origins. The unique properties of this peptide and equivalent peptides can contribute toward the development of diverse human differentiating cell sources for biomedical and research purposes.

SEQUENCE LISTING

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Claims:

1. A cell growth and/or differentiation regulatory peptide comprising a sequence of about 9 to about 150 amino acids derived from Acetylcholinesterase amino acid sequence.
2. A peptide according to claim 1, characterized in that the sequence contains a region predicted to be rich in beta-pleated sheet structure and turns.
3. A peptide according to claim 1, characterized in that the sequence contains a predicted amphipathic helix structure.
4. A peptide according to claim 1, characterized in that the sequence is derived from the C-terminal region of acetylcholinesterase.
5. A peptide according to any one of claims 1 to 3, characterized in that the sequence is derived from the readthrough or synaptic variant of acetylcholinesterase.
6. A peptide according to claim 4, characterized in that the sequence is derived from the mature form of acetylcholinesterase.
7. A peptide according to claim 5, characterized in that the sequence is about 20 to about 50 amino acids in length.
8. A peptide according to claims 1 to 6, characterized in that the sequence is SEQ ID No. 1, SEQ ID No. 2, or SEQ ID No. 3.
9. A peptide according to claim 7 which is SEQ. ID No. 1, SEQ. ID. No. 2, or SEQ ID No. 3.
10. A peptide according to claim 1 which is cyclic.

11. A synthetic peptide comprising the amino acid sequence of SEQ ID No. 1, SEQ ID No. 2, or SEQ ID No. 3.

12. A synthetic peptide according to claim 1 having the amino acid sequence denoted by SEQ ID No. 1, 2 or 3.

13. A linear synthetic peptide according to claim 11 or 12.

14. A cyclic synthetic peptide according to claim 11 or 12.

15. A peptide of claim 1 capable of promoting cell survival and/or differentiation comprising the amino acid sequence denoted by SEQ ID No. 1, SEQ. ID. NO. 2 or SEQ ID No. 3, and functional analogues and derivatives thereof.

16. A peptide according to claim 1 which is a hematopoietic stem cell growth and/or differentiation regulatory peptide.

17. A peptide of claim 16 capable of promoting stem cell survival and/or myeloid and megakaryocytic differentiation comprising the amino acid sequence denoted by SEQ ID No. 1, SEQ. ID. NO. 2 or SEQ ID No. 3, and functional analogues and derivatives thereof.

18. A peptide according to any one of claims 15 to 17 being a synthetic peptide.

19. A cyclic peptide according to claims 15 to 18.

20. Pharmaceutical compositions comprising a synthetic peptide of any one of the preceding claims.

21. Pharmaceutical compositions comprising a synthetic peptide comprising the amino acid sequence of SEQ ID No. 1, SEQ. ID. NO. 2, or SEQ ID No. 3.

22. A pharmaceutical composition according to claim 21 wherein said peptide has the amino acid sequence denoted by SEQ ID No. 1, SEQ. ID. NO. 2, or SEQ ID No. 3.

23. A pharmaceutical composition according to claim 21 or claim 22 wherein said peptide is a cyclic peptide.

24. A pharmaceutical composition for regulating hematopoietic stem cell growth comprising a peptide of any one of claims 1 to 19.

25. A pharmaceutical composition according to claim 24 for use in promoting survival of stem cells.

26. A pharmaceutical composition according to claim 24 for use in promoting differentiation of stem cells.

27. A pharmaceutical composition according to claim 24 for use in promoting growth of stem cells.

28. A pharmaceutical composition according to claim 24 for use in promoting the growth-enhancing effect a growth factor on stem cells.

29. A pharmaceutical composition according to claim 24 for use in promoting the growth-enhancing effect of GM-CSF, SCF, or TPO on stem cells.

30. A pharmaceutical composition according to claims 25-29, wherein the stem cells are embryonic stem cells.

31. A pharmaceutical composition according to claims 25-29, wherein the stem cells are nerve stem cells.

32. A pharmaceutical composition according to claims 25-29, wherein the stem cells are epithelial stem cells.

33. A pharmaceutical composition according to claims 25-29, wherein the stem cells are mesenchymal stem cells.

34. A pharmaceutical composition according to claims 25-29, wherein the stem cells are hematopoietic stem cells.

35. A pharmaceutical composition comprising a peptide according to claims 1 to 19 for use in the treatment of thrombocytopenia, post-irradiation condition, post-chemotherapy condition, or condition following massive blood loss.

36. A pharmaceutical composition comprising a peptide according to claims 1 to 19 for use in inducing synthesis of acetylcholinesterase mRNA.

37. A pharmaceutical composition according to any one of claims 21 to 23 for use in promoting the formation of hematon bodies.

38. An antibody directed against a peptide of claims 1 to 19, for use in diagnosing elevated glucocorticoid level; bone marrow stress, abnormality, dysfunction, or stressed condition, or of increased platelet count or of brain infarct risk in a mammal.

39. The antibody of claim 38, wherein the peptide is a peptide of claim 9.

40. A method for the diagnosis of elevated glucocorticoid level; bone marrow stress, abnormality, dysfunction or stressed condition, or of increased platelet count or of brain infarct risk in a mammal, comprising obtaining a sample from said mammal, contacting said sample with an antibody of claims 38 or 39, removing unbound antibody, and detecting the extent of reaction between said antibody

and acetylcholinesterase or a fragment thereof present in said sample.

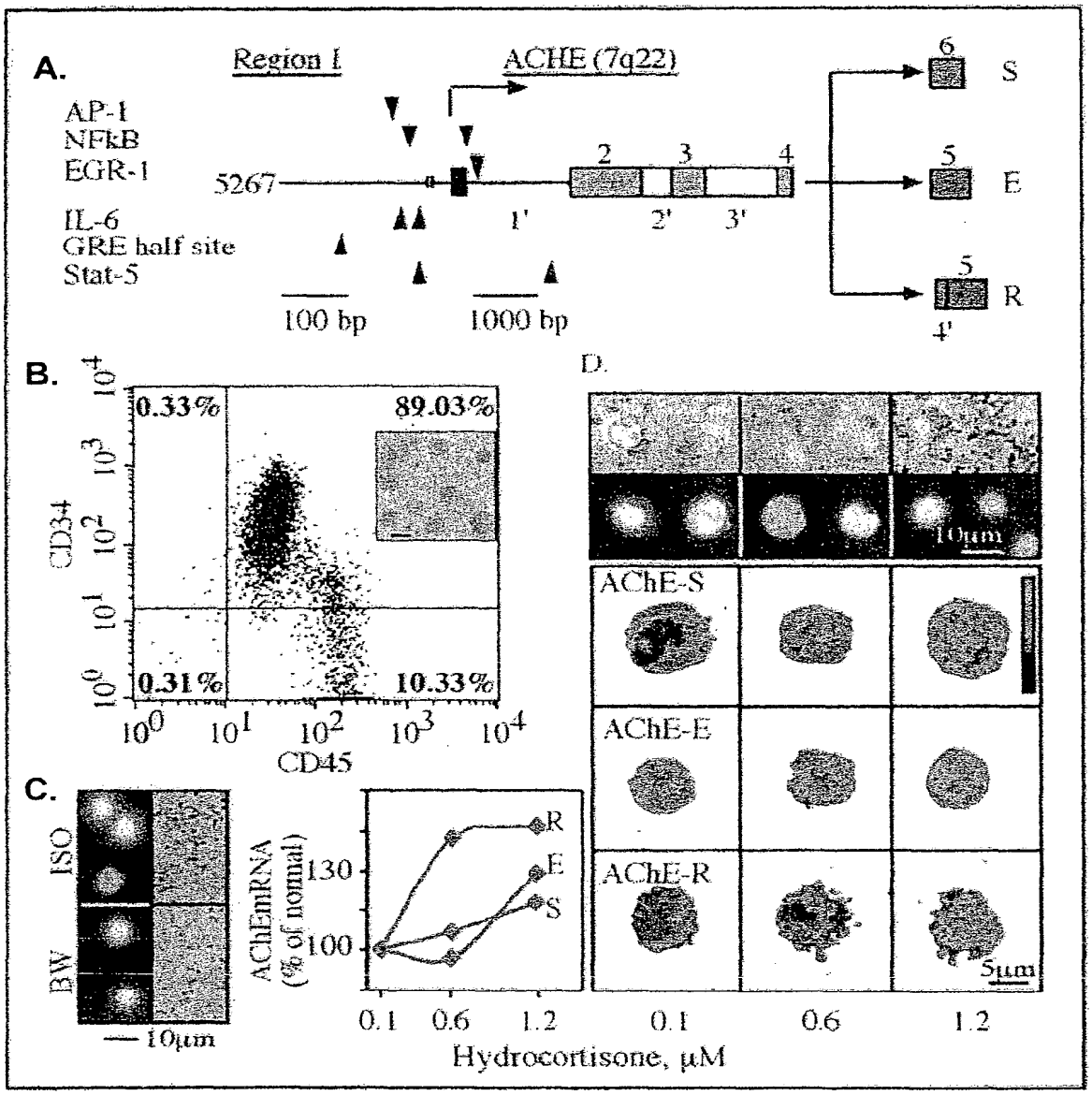
41. A method of claim 40 wherein said sample is a serum or bone marrow sample.

42. A peptide of claims 1-19 for use in *ex vivo* expansion of hematopoietic stem cells.

43. A peptide of claims 1-19 for use in *in vivo* expansion of hematopoietic stem cells.

44. A peptide of claims 1-19 for use in *ex vivo* or *in vivo* promotion of megakaryocytic differentiation of hematopoietic stem cells.

Figure 1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4'
S
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CD34
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0.31%
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C.
ISO
BW
10μm
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5μm

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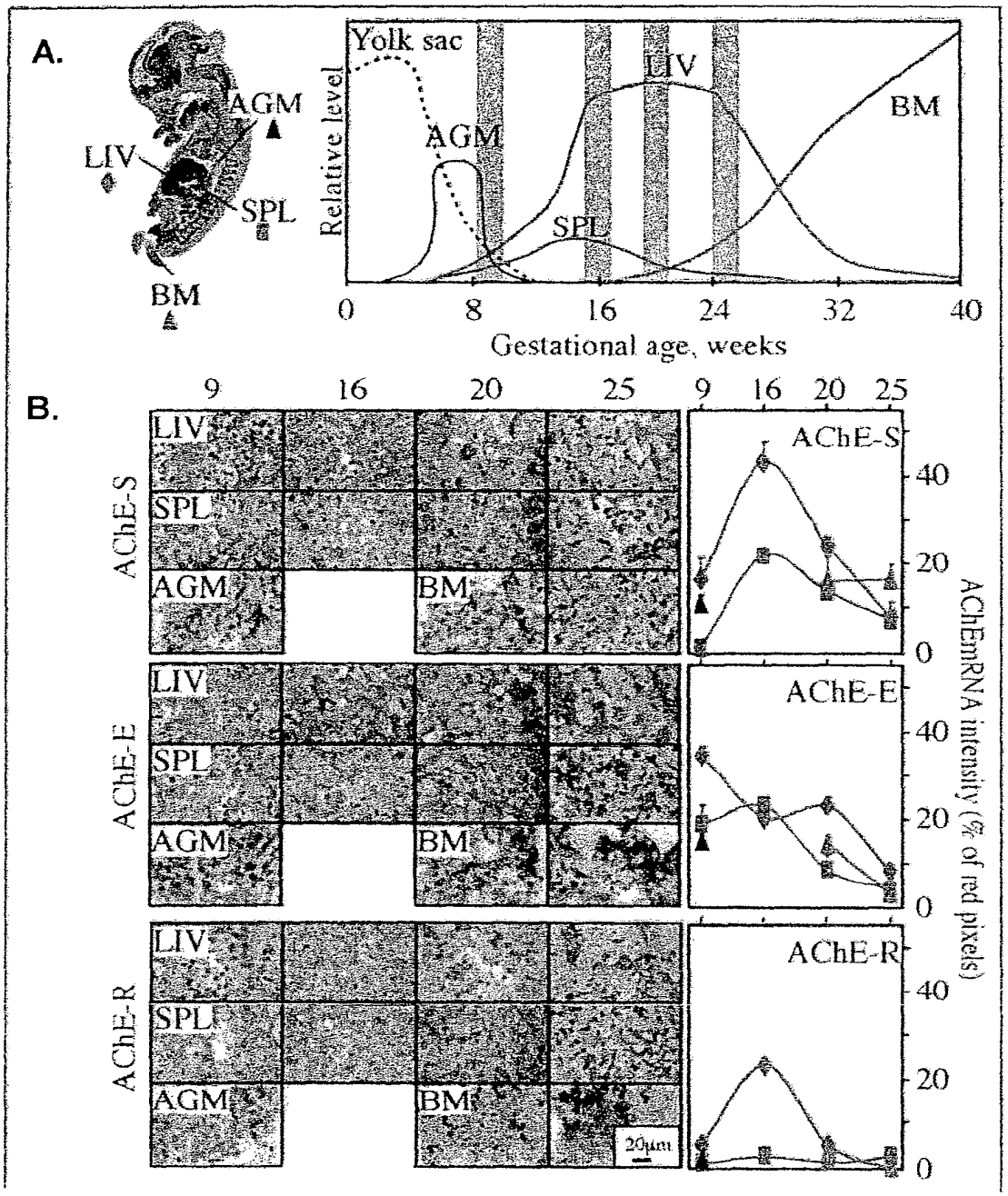
Figure 2



העתק מתאים למקור

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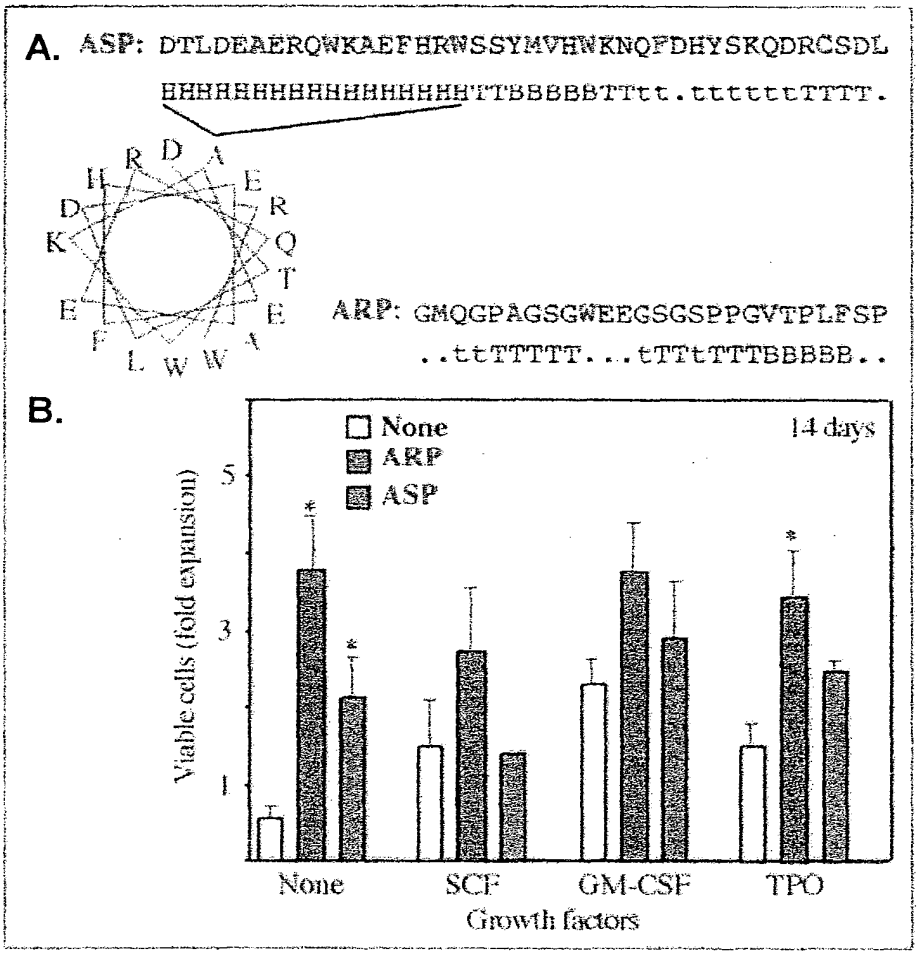
Figure 3



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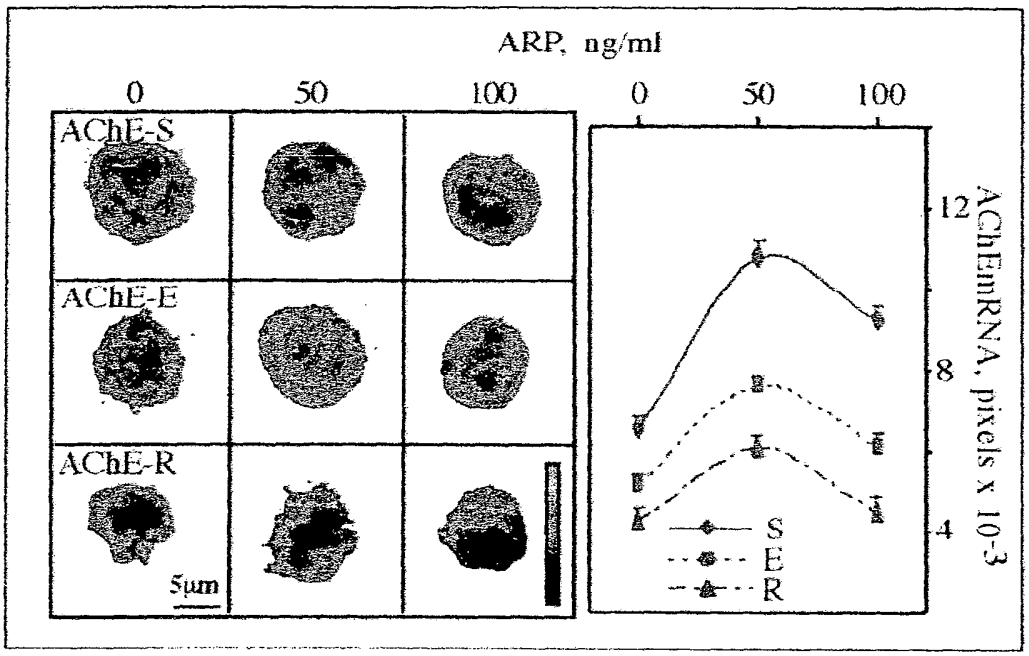
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E
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Figure 5

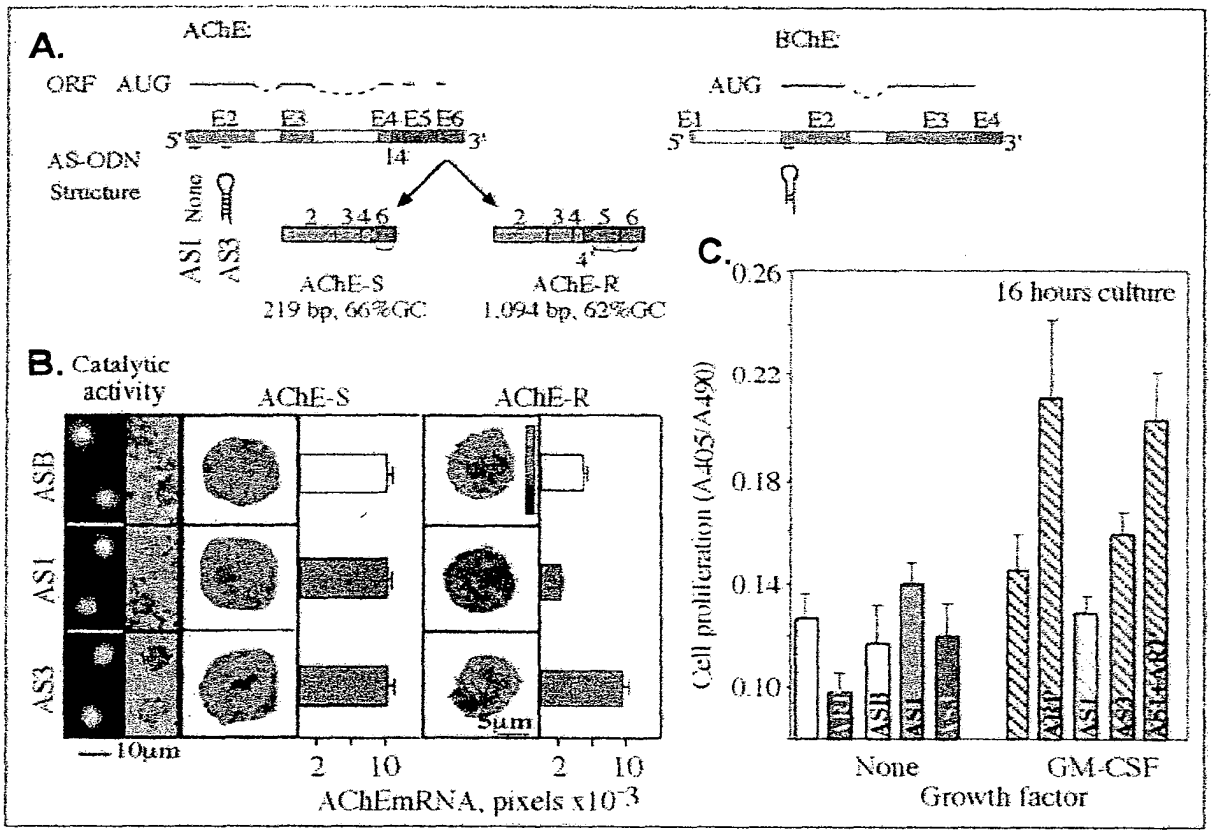

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Figure 6

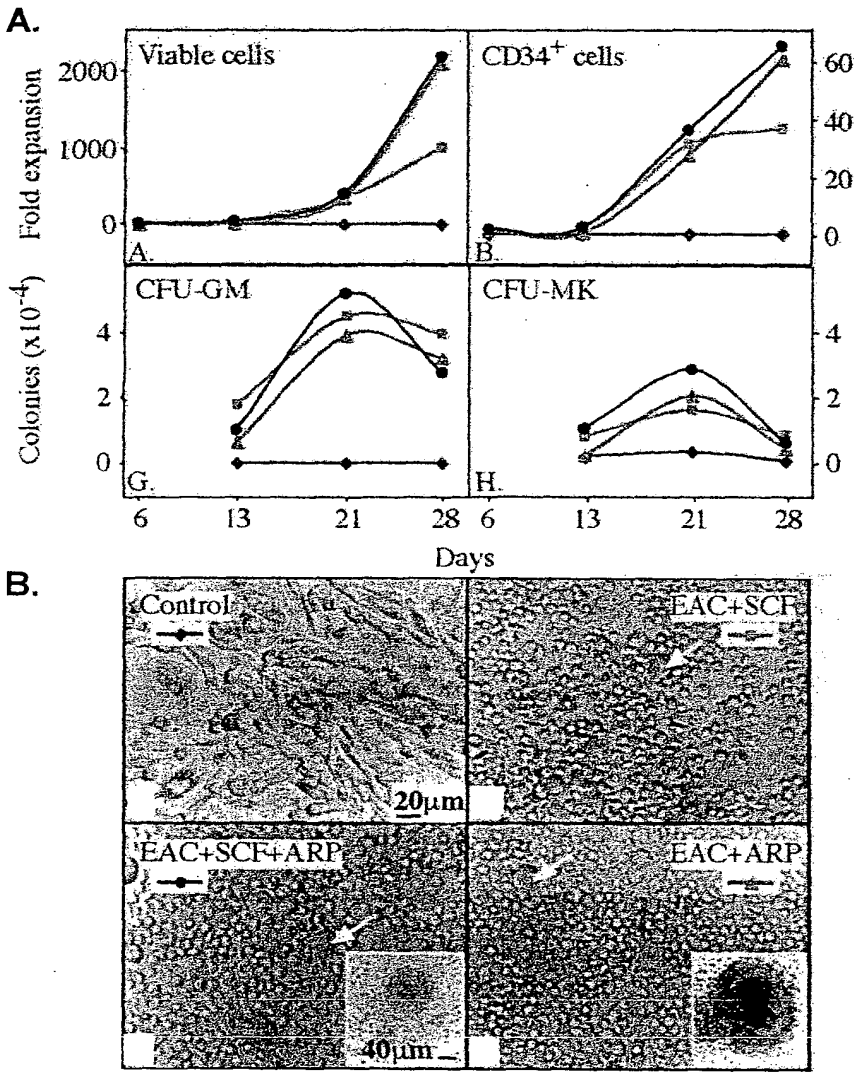

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Figure 7